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DISK-SPHERE TRANSFORMATION IN MAMMALIAN RED CELLS

II. THE NATURE OF THE ANTI-SPHERING FACTOR

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(With Four Text-figures)

THE transformation of red cells from disks to spheres in a drop of a saline suspension between slide and coverglass (Ponder, 1929, 1934) has recently been shown by one of us (Furchgott, 1939) to be the result of two changes. The first is a rise in the *p*H of the suspension above 9·2 because of the alkaline nature of the glass surfaces. The second is an adsorption out of the suspension by the glass surfaces of a substance which, if present, prevents the formation of spheres at the *p*H attained between slide and coverglass. The determination of the chemical nature of this substance, termed the "anti-sphering factor", and experimental attempts to gain an insight into the physical mechanism of the phenomena of shape changes are the subject of this paper.

I. THE METHOD AND THE SCALE OF ANTI-SPHERING ACTIVITY

As previously noted (Furchgott, 1939), quantities of cells, sufficient to work with, cannot be completely freed of the anti-sphering factor by thorough washing alone, but can be freed completely if they are treated with large quantities of clean glass beads, which adsorb the anti-sphering factor from the cells. In the removal of the factor from the cells for the experiments reported here, the cells were first washed free of their serum. They were then suspended in 1% saline in a concentration of about 1 part in 800, and this suspension was evenly poured into a beaker full of glass beads. The beads were 3 mm. in diameter and afforded about 10 sq. m. of surface area for every 0·5 c.c. of cells. After standing about 5 min. with the beads, the cell suspension was decanted through cheese cloth. The cells were then centrifuged down and resuspended in a concentration of about 1 part in 400 in fresh saline.

In testing for the anti-sphering activity of serum, a given amount was added to

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[1] John D. Jones Scholar at the Biological Laboratory, 1939.

5 c.c. of the treated, factor-free suspension, and then one or two drops of 0·15 N NaOH were added to the suspension so that the pH suddenly rose from around neutrality to between 10·2 and 10·7. After waiting about 5 min. to allow for the passing of the temporary tendency toward sphere formation (discussed in the previous paper), the shape of the cells at the high pH was observed in a drop in the concavity of a hanging-drop slide. If sufficient anti-sphering factor had been added to the suspension, the cells were smooth biconcave disks. If none or extremely little had been added, the cells were smooth spheres. If an intermediate amount had been added, the cells were intermediate crenated forms. All tests for anti-sphering activity with a given bead-treated suspension were performed within an hour of the bead treatment, because the cells on somewhat longer standing showed less and less response to solutions containing anti-sphering factor.

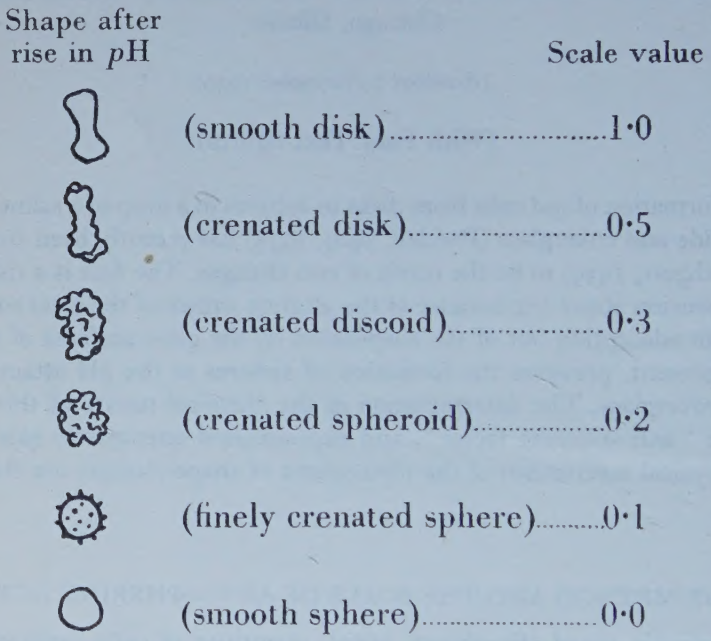
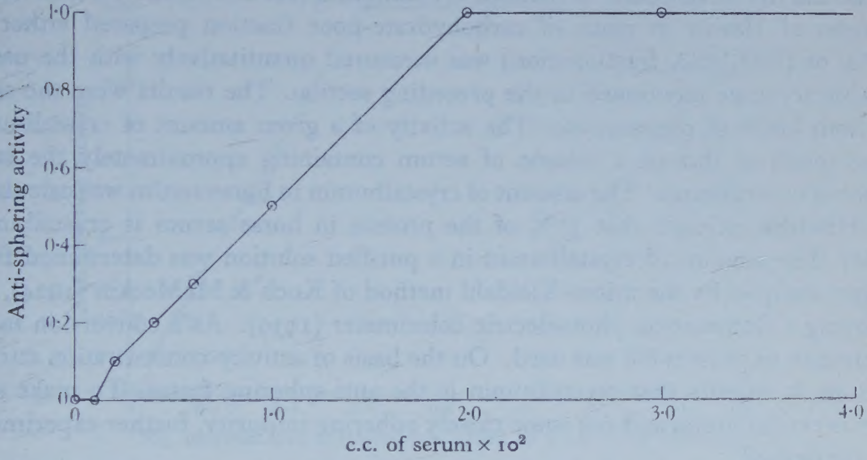

Shape after rise in pH
Scale value
(smooth disk)........1·0
(crenated disk)........0·5
(crenated discoid)........0·3
(crenated spheroid)........0·2
(finely crenated sphere)........0·1
(smooth sphere)........0·0

Fig. 1. Arbitrary scale of anti-sphering activity.

In the attempt to determine what substance normally contained by serum and cells is the anti-sphering factor, it was found useful to set up an arbitrary semi-quantitative scale of anti-sphering activity. With this scale the activity of a given amount of serum and a given amount of some fraction separated from serum could be compared, and the activity shown by a given amount of serum could be approximately measured by the shape of cells in the 5 c.c. of previously factor-free suspension to which the serum had been added and the pH of which had been raised to about 10·5. The arbitrary activity values corresponding to the different shapes are given in Fig. 1. If the shapes varied in the same suspension, the predominating shape was used.

Using this arbitrary scale, the anti-sphering activity of horse serum is plotted gainst the quantity of horse serum in Fig. 2. If any single substance fractionated rom horse serum is the anti-sphering factor, it should show just as much activity er given amount as that volume of serum containing the same amount of the ubstance, i.e. its concentration-activity curve should be the same as the curve in ig. 4.

As pointed out in the previous paper (Furchgott, 1939), cells and sera of different nammalian species are interchangeable so far as the anti-sphering factor is con-erned. That is, human serum furnishes anti-sphering factor for dog cells, horse erum for human cells, etc. This was fortunate in the present work, for horse serum as the most suitable for fractionation, while human cells were the most readily vailable in a fresh condition. All the suspensions used were therefore prepared om freshly drawn human cells.



ig. 2. Anti-sphering activity (ordinate) in a suspension of 5 c.c. of cells previously freed from the factor as a function of the volume of horse serum (abscissa) added to the suspension.

II. THE PROTEIN NATURE OF THE ANTI-SPHERING FACTOR

Several preliminary experiments indicated that the anti-sphering factor is a rotein. The factor was adsorbed from cells and from serum on to glass surfaces as a rotein might be. The anti-sphering activity of the serum was destroyed by heating ie serum in a boiling water bath. When all the protein in serum was precipitated y saturation with Na_2SO_4, the supernatant fluid had no anti-sphering activity, hile a solution of the precipitate had very much. If the precipitate was extracted ith lipoid solvents, it did not lose its activity.

To obtain the various fractions of the serum proteins for the purpose of testing ieir separate activities, a fractionation of horse serum with Na_2SO_4 was carried out cording to the method of Kekwick (1938). The total globulin fraction showed no tivity, while the total albumin fraction showed a large amount of activity. On separating the albumin fraction into a carbohydrate-rich and a carbohydrate-poo fraction, it was found that the carbohydrate-poor fraction had much more activit than the carbohydrate-rich fraction. A fractionation of the albumin fraction of hors serum was also carried out with $(NH_4)_2SO_4$ according to the method of Hewit (1938*a*). Here again the carbohydrate-poor fraction, the crystalbumin of Hewitt was extremely active, while the carbohydrate-rich fraction, the seroglycoid o Hewitt, had very little activity. This slight activity of the carbohydrate-rich fraction can probably be attributed to small amounts of the carbohydrate-poor fraction present as an impurity.[1]

The activity of solutions of purified crystalbumin (for convenience we are using this term of Hewitt in place of carbohydrate-poor fraction prepared either by Na_2SO_4 or $(NH_4)_2SO_4$ fractionation) was measured quantitatively with the use o the arbitrary scale mentioned in the preceding section. The results were the sam with both kinds of preparations. The activity of a given amount of crystalbumin was as much as that of a volume of serum containing approximately the same amount of crystalbumin. The amount of crystalbumin in horse serum was calculate from Hewitt's estimate that 33% of the protein in horse serum is crystalbumin (1938*b*). The amount of crystalbumin in a purified solution was determined from nitrogen analyses by the micro-Kjeldahl method of Koch & McMeekin (1924), bu employing a Summerson photoelectric colorimeter (1939). As a conversion factor for nitrogen to protein 6·6 was used. On the basis of activity-concentration curves therefore, it appears that crystalbumin is the anti-sphering factor. To make sure that it is crystalbumin and not some closely adhering impurity, further experiments were performed.

III. FURTHER EVIDENCE THAT THE FACTOR IS CRYSTALBUMIN

(1) *A denaturation experiment.* If the anti-sphering activity of crystalbumin were due to an impurity, it might well happen that the rate of heat denaturation o the crystalbumin would be different from the rate of heat inactivation of the active impurity. If this were the case, the curve for activity of partially denatured solutions of crystalbumin against concentration of undenatured protein remaining afte heating would not be the same as the activity-concentration curve for unheated solutions of crystalbumin. However, if the curves were the same, the possibility o crystalbumin being the factor would be strengthened.

The solution of crystalbumin used contained about 0·5 mg. N per c.c. It wa made isotonic by the addition of NaCl. Two c.c. portions were put into seve 15 c.c. conical centrifuge tubes. These tubes were then dipped for varying period from a few seconds to a couple of minutes into boiling water. The amount of hea denaturation depended on the time of heating. After the tubes had been cooled i the ice-box for half an hour, the coagulated denatured protein in them was centri fuged down and the supernatant solutions, containing the undenatured protein were decanted off. One c.c. of each solution was analysed for nitrogen. Five-tenths of a c.c. of each was tested for anti-sphering activity.

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[1] A sample of purified $(NH_4)_2SO_4$ fractionated seroglycoid furnished us by Dr Hans Neurath c Duke University Medical School had no anti-sphering activity at all.

In Fig. 3 are plotted the results of a few such experiments, using the arbitrary scale for activity. Also in the figure are points obtained by testing the activity of various amounts of a solution of unheated purified crystalbumin. In view of the arbitrary nature of the scale, the points obtained with undenatured solutions and partially denatured solutions lie sufficiently close to the same concentration-activity curve to be evidence against the anti-sphering activity of crystalbumin being the result of an impurity.

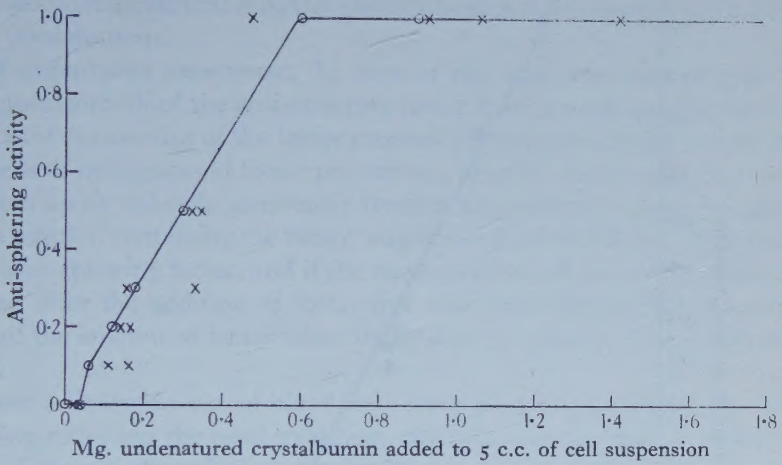

Fig. 3. Anti-sphering activity of undenatured (circles) and partly denatured (crosses) crystalbumin solutions as a function of the undenatured crystalbumin present.

(2) *An electrophoresis experiment.* Although it is impossible to free working quantities of cells of anti-sphering factor by washing, there must be, as pointed out in the last paper, a distribution of factor between the cells and the saline of the washing fluid with the equilibrium greatly in favour of the cells. Micro-Kjeldahl's were done on the saline washing fluids of cells previously washed free of serum, but the traces of nitrogen found were too small to be quantitatively determined. We decided to try to adsorb what little anti-sphering factor there might be in the saline washing fluids on to small amounts of quartz particles. The adsorption, if it occurred, could be detected by electrophoresis of the particles. Moreover, if the pH-mobility curve of the quartz particles treated with saline washing fluids was the same as that obtained with quartz particles coated with purified crystalbumin, it would be strong evidence that the anti-sphering factor is that protein.

The cells, generally from about 1 c.c. of fresh human blood, were first washed three times with 50 c.c. portions of saline. After each centrifuging practically all of the supernatant washing fluid was removed by a suction pipette, so that after the third washing the protein remaining from the original serum was negligible. The cells were then mixed with 50 c.c. of saline for the fourth washing, and after about 10 min. standing, the cells were centrifuged down. About 25 c.c. of the supernatant saline were then decanted into another centrifuge cup, leaving all of the cells in the first cup. To the decanted supernatant was added about 25 mg. of finely powdered quartz. The mixture was allowed to stand for several hours, with stirring about every 10 min. The quartz particles were then centrifuged down and washed once with water before being tested in the electrophoresis cell.

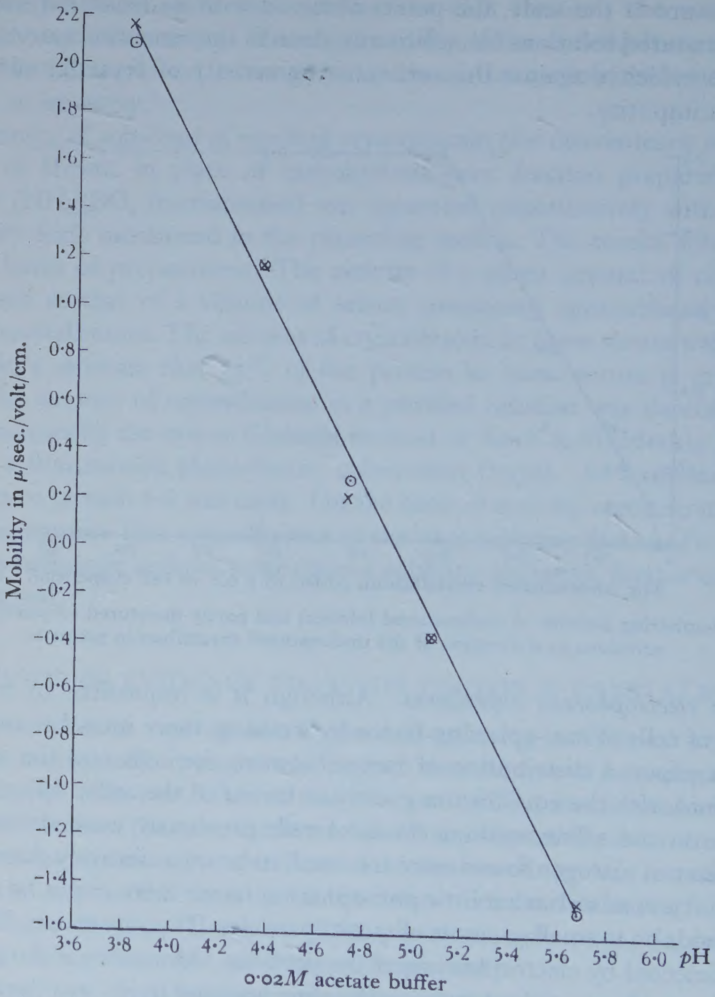

Fig. 4. Electrophoretic mobility in 0·02 *M* acetate buffer at 25° C. of quartz particles coated with crystalbumin (circles) and with protein from the supernatant washing fluid of serum-free red cells (crosses).

Since human cells were being used, the nature of this experiment demanded that human crystalbumin be used also. A preparation of it was made by the Na_2SO_4 fractionation of Kekwick (1938). Quartz particles were coated with it by mixing them with a solution of the preparation. These particles were also washed once with water before being tested in the electrophoresis cell.

The electrophoresis cell was of the Abramson type (1934). The technique used was that described by Abramson and more recently by Moyer (1936). The suspending solutions used in obtaining the *p*H-mobility curves of the particles were 0·02 *M* acetate buffer. The results of the electrophoretic measurements are shown in Fig. 4. Each point is the average of measurements on at least two different preparations.

The points for the particles coated by treatment with the saline wash agree remarkably well with those for the particles coated with purified crystalbumin. If anti-sphering factor is the substance adsorbed from the saline washing fluid, and evidence would indicate that it is, this electrophoresis experiment is further evidence that it is crystalbumin.

(3) *A quantitative experiment.* In view of the very large area of glass surface required to adsorb all of the anti-sphering factor from a small quantity of red cells, it looked as if the amount of the factor retained by washed red cells was of the order of hundreds of milligrams of factor per 100 c.c. of cells. Conversely, the amount of factor taken up by red cells, previously freed of anti-sphering factor, on addition of them to a solution containing the factor, might also be of that order. If crystalbumin were the anti-sphering factor, and if the concentrations of protein in a solution of it before and after the addition of factor-free cells were determined, a quantitative estimate of the amount of factor taken up by a given quantity of red cells might be obtained.

The red cells from 1 c.c. of blood were freed of anti-sphering factor. After the centrifuging following the bead treatment, the cells were suspended in about 6 c.c. of saline. Fifty c.c. of a solution of crystalbumin (about 0·07%) in 1% saline were placed in a centrifuge cup. Five c.c. of this solution were removed and analysed quantitatively for nitrogen. Then, to replace this 5 c.c. in the centrifuge cup, 5 c.c. of the cell suspension were added. The 50 c.c. of suspension now in the cup were stirred by rotation of the cup for 5 min. The cells were then centrifuged down, and two 5 c.c. samples were removed from the cell-free supernatant fluid and analysed for nitrogen. A count was made on the cell suspension of which 5 c.c. had been added to the crystalbumin solution. From this count, assuming that a count of five million correspond to 42% of cells by volume, we calculated the volume of cells and of solution added to the 45 c.c. of crystalbumin solution. From the total volume of supernatant fluid and the analyses made on aliquot portions of it, we calculated the total nitrogen content of the supernatant fluid. The theoretical total nitrogen content of the supernatant fluid, if the cells had taken up no protein, would have been 0·9 of the total content of the original 50 c.c. of crystalbumin solution. From the difference between the theoretical content and the content actually found by analysis, we estimated how much protein (using $6 \cdot 6 \times N$ to give the protein) was taken up by a given quantity of red cells.

The results of four such experiments are included in Table I. The rather large variations in the figures for grams of crystalbumin taken up per 100 c.c. of cells in the individual experiments may be attributed to the limitations of the micro-Kjeldahl analysis with such small amounts of nitrogen as were analysed for here (of the order of 0·5 mg.). However, the results undoubtedly show that the cells take up a considerable quantity of crystalbumin—a quantity of the order of 800 mg. per 100 c.c. of cells.

Table I

1 Nitrogen content of original 50 c.c. of crystalbumin solution	2 Volume of cells in 5 c.c. of suspension added to 45 c.c. of crystalbumin solution	3 Theoretical nitrogen content of supernatant solution after addition of cells	4 Actual nitrogen content of supernatant solution from nitrogen analyses	5 Amount of crystalbumin taken up by cells (6·6 × difference between columns 3 and 4)	6 Amount of crystalbumin taken up by 100 c.c. of cells
mg.	c.c.	mg.	mg.	mg.	g./100 c.c.
5·32	0·24	4·79	4·57	1·45	0·61
5·89	0·25	5·30	4·93	2·44	0·98
5·42	0·18	4·88	4·70	1·19	0·66
6·48	0·17	5·83	5·59	1·58	0·93
				Average	0·80

Removal of crystalbumin from a solution by red cells previously freed of anti-sphering factor.

IV. ELECTROPHORESIS EXPERIMENTS

With the hope of finding out something about the physical mechanism of the phenomena of shape changes in red cells we made electrophoretic measurements on cells treated in the various ways described in this paper and in the previous one (Furchgott, 1939). If changes in the exterior surface of the cell were related to the shape changes, such changes might be detected by changes in electrophoretic mobility. The method of determining the electrophoretic mobilities of the cells was the same as used for determining those of the coated quartz particles, discussed earlier in this paper.

The first set of measurements was made on cells in $M/15$ phosphate buffer of *p*H 7·4 and of specific resistance of 126 Ω at 25° C. Measurements were made on washed cells containing anti-sphering factor, on cells freed of anti-sphering factor with glass beads, and on washed cells of which the activity of the anti-sphering factor had been counteracted by first shaking a suspension of the cells against the palm of the hand. This counteracting of anti-sphering activity, as pointed out in the previous paper (Furchgott, 1939) is probably due to fatty acids coming from the palm. Measurements were also made on ghosts which had been put through the temporary sphering process discussed in the previous paper. These ghosts, made by hypotonic lysis, were sphered by suddenly raising the *p*H of a suspension of them in isotonic saline to *p*H 9·5. After a few minutes, during which the spheres changed back to disks and discoids, the *p*H was readjusted to neutrality, and the ghosts were centrifuged down for the electrophoretic measurements.

The results of the measurements in phosphate buffer are included in Table II. The mobilities (all corrected to 25° C.) of the differently treated cells are easily within experimental error of one another. They agree very well with Abramson's

1934) figure of 1·31 μ/sec./volt/cm. for the mobility of human cells in the same hosphate buffer solution.

The cells in the phosphate buffer were all discoidal. After finding no differences n their mobilities in that buffer, we decided to make measurements at a *p*H high nough to actually cause the cells to be either disks or spheres, depending on the reatment given them. For this purpose a glycine buffer was made up containing ·5 parts of a glycine and NaCl solution (7·505 g. glycine and 5·85 g. NaCl per litre) o 4·5 parts of 0·1 *N* NaOH. To 10 c.c. of a mixture of 1 part of this buffer with parts of 1% saline was added a small amount of cells for electrophoresis measurenents plus 0·05 c.c. of either a dilute solution of crystalbumin or distilled water, lepending on the treatment desired. The *p*H of the resulting suspensions was 10·1 nd the specific resistance at 25° C. was 66·7 Ω. Measurements were made on pherical cells deprived of anti-sphering factor by glass beads, on smooth discoidal ells, resulting from the presence of the crystalbumin added to the buffer, and on vashed cells which were spheres in the buffer because a suspension of them had reviously been shaken against the palm of the hand.

Table II

Conditions of cells	Mobility in μ/sec./volt/cm.
Cells containing the factor	1·30
Cells freed of the factor	1·29
Cells with activity of factor counteracted by fatty acid	1·30
Ghosts after temporary sphering	1·31

Electrophoretic mobility at 25° C. of differently treated human red cells in *M*/15 phosphate uffer at *p*H 7·4.

Table III

Condition of cells	Shape of cells	Mobility in μ/sec./volt/cm.
Cells containing the factor	Smooth disks	1·04
Cells freed of the factor	Smooth spheres	1·07
Cells with activity of factor counteracted by fatty acid	Smooth spheres	1·04

Electrophoretic mobility at 25° C. of differently treated human red cells in a saline-glycine buffer olution of *p*H 10·1.

The results of these measurements are given in Table III. Again the mobilities f the differently treated cells are within experimental error of one another.

To complete the electrophoresis experiments we investigated the mobility of ells made spherical by the addition of small traces of lecithin (Ponder, 1936). he suspending medium was a solution of 5 parts of 1% saline to 1 part of /15 phosphate buffer of *p*H 7·4 and it had a specific resistance at 25° C. of 4·4 Ω. Here again the mobilities of spherical and non-spherical forms were within xperimental error of one another: 1·00 μ/sec./volt/cm. for the disks, and ·98 μ/sec./volt/cm. for the cells sphered by lecithin.

V. DISCUSSION

This investigation appears to identify the anti-sphering factor definitely a crystalbumin, yet it does nothing, in a positive sense, toward clarifying the physica mechanism behind the phenomena of shape changes in red cells. If anything, i leaves the situation more puzzling than ever. The red cells take up crystalbumin in an amount of the order of 800 mg. per 100 c.c. of cells; such an amount is enough to form more than one monomolecular layer on the surface of the cells, yet from th electrophoresis data one can see that the exterior surface of the cells, which control their electrophoretic mobility, definitely remains the same whether the cells have o have not taken up crystalbumin. Whether the albumin molecules go below th exterior surface of the cell membrane cannot be said.[1]

The electrophoresis experiments probably rule out surface tension changes as a possible explanation of the shape-change phenomena. The constancy of the mobility of cells put through the various treatments discussed here is in keeping with th constancy of mobility found for ghosts made by various means of haemolysis (Abramson, Furchgott & Ponder, 1939).

From the electrophoretic data it appears that spherical cells have the same charg per unit area of surface as the discoidal cells. This points to the conclusion that th transformation to the spherical form does not involve the compression of an exterio molecular film into a more condensed film. Rather, it may indicate a very fine submicroscopic crenation of the periphery of the cell in the spherical form, this being the culmination of the successively smaller microscopic crenations found in the intermediate forms between disk and sphere.

We cannot say how fatty acids counteract the action of the crystalbumin. Perhaps the molecules of the fatty acid compete with the protein molecules for certain positions in the membrane, in which the protein molecules may have t be if they are to exert their anti-sphering action.

VI. SUMMARY

1. Experimental evidence has been presented showing that crystalbumin (the carbohydrate-poor fraction of serum albumin) is the factor which prevents mammalian red blood cells from becoming spherical at *p*H values over 9·2.
2. The amount of crystalbumin taken up from a solution of it by red cells previously freed of it is of the order of 800 mg. per 100 c.c. of red cells. If this amount is all taken up at the red cell surface, it would form a layer only a few molecules thick.

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[1] If all the crystalbumin taken up by the cells is supposed to be at the cell surface, it would form a layer of 45–60 A thick (assuming a density of unity and excluding possible contribution of water). Calculated on the same basis, the thickness of the red cell envelope is only about 120 A (Fricke Parker & Ponder, *J. Cell. and Comp. Physiol.* **13**, 69, 1939), so the layer of crystalbumin would b from one-third to one-half the thickness of the cell's surface structure, but only a few molecule thick.

3. The electrophoretic mobility in phosphate buffer of *p*H 7·4 is the same for ells containing crystalbumin, cells free of crystalbumin, cells with anti-sphering ctivity counteracted by fatty acid, and ghosts which have been temporarily sphered y a rise in *p*H. The mobilities in a saline-glycine buffer solution of *p*H 10·1 for the irst three classes of cells just mentioned are also the same. The mobility of cells phered with lecithin in a saline-phosphate buffer solution is the same as that for ntreated discoidal cells.

We wish to thank Dr Henry B. Bull of Northwestern University Medical chool for his interest and for helpful suggestions relative to this work.

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THE RESPONSE OF THE WATER-REGULATING MECHANISM OF DEVELOPMENTAL STAGES OF THE COMMON TOAD, *BUFO BUFO BUFO* (L.), TO TREATMENT WITH EXTRACTS OF THE POSTERIOR LOBE OF THE PITUITARY BODY

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(With Two Text-figures)

Investigations made in recent years have demonstrated a connexion between the posterior lobe of the pituitary gland and water regulation in Amphibia. Injection of extracts causes a retention of water in the body of frogs (Brunn, 1921; Heller 1930; Steggerda, 1931; Collin & Drouet, 1932; Steggerda & Essex, 1934; Oldham 1936; Boyd & Brown, 1938; Boyd & Whyte, 1939), toads (Steggerda, 1931; Novelli 1933), Amblystoma (Bělehrádek & Huxley, 1927), *Necturus maculatus* (Steggerda 1937), and some reptiles (Boyd & Dingwall, 1939). In mammals the antidiuretic effect of the posterior lobe is now well established. On the other hand, freshwater fish show no such effect even when given doses of pituitrin up to 2·0 I.U. per 10 g body weight. Nevertheless, the fish pituitary contains a substance causing a typical water retention in frogs (Boyd & Dingwall, 1939). It appears therefore that while the principle concerned is elaborated in the fish pituitary, either the appropriate effector is not developed or an alternative system which deals with the greater part of water regulation, is present. Thus, on the basis of the data available, it may be said that the regulation of the body water by agents of the posterior pituitary is characteristic of terrestrial but not of primarily aquatic vertebrates.

The Salientia during development undergo a transition from an aquatic to a terrestrial mode of life and it seemed a matter of some interest to determine whether Amphibia exhibited the adult response from an early stage or whether this was acquired during metamorphosis. In the latter case, interesting information would be obtained on the adjustments undergone by individual animals passing from an aquatic to a terrestrial habitat and on the larger problem of the mechanisms involved in the invasion of land by vertebrates. For this purpose, the effect on body water of extracts of the posterior pituitary has been investigated on a series of developmental stages of the common toad.

MATERIAL AND METHODS

Recently hatched tadpoles of *Bufo bufo bufo*, having well-developed ventral
ıckers, were collected on 3 May and placed in large bowls containing tapwater
ıd plants of *Elodea canadensis*. They were fed each morning and evening on
owdered wheat germ and the water was changed twice weekly. A piece of liver
r beef was suspended in each bowl for about 6 hr. daily from 15 May onwards.
'adpoles in which one or both forelimbs had emerged were transferred to glass jars
ontaining a little water and were fed on *Drosophila* spp.

The toads continued to develop normally and individuals were taken from stock
nd used in experiments between 30 May and 25 August, when the last had meta-
ıorphosed. Observations were made on all stages from the appearance of the
indlimb bud until immediately after metamorphosis.

Tadpoles of the common toad survive treatment involving injection and con-
iderable handling much better than do those of *Rana t. temporaria*. Their skin is
ougher and precise quantities can be injected into this species more readily since
ne body wall is flaccid and the coelomic fluid does not gush out when the skin is
ierced. On the other hand, the consistent succession of external changes associated
vith metamorphosis in the frog (Etkin, 1932) is not found in the toad. For instance,
ne emergence of the last forelimb is frequently delayed until the tail has nearly
een absorbed, and again, the loss of the horny jaws and final development of the
oad-like mouth may take place at any time during this process. For this investiga-
ion, a developmental schedule, closely correlated with time except in the case of
ertain of the three-limbed forms, was established by neglecting the finer changes
nd referring only to limb development and tail absorption (Table I, left-hand
olumn). An extra stage was added as it was observed that after tail absorption
vas complete, the toad remained a deep greenish brown (*dark toad*) for about 30 hr.
efore suddenly assuming the adult colour (*yellow toad*).

Two types of experiment were made to test the response of the developmental
tages of the toad to posterior lobe extracts. First, adult 20 g. *Bufo b. bufo* were
njected with extracts and the effect on body water was followed by weighing the
nimals immediately before injection and afterwards at intervals for 8 hr. The
nimals had access to water. The adults behaved similarly to other Salientia, showing
n increase up to about 40 % of their initial weight 2–3 hr. after treatment, followed
y a return to normal. Since the animals were given no food, this weight change
as been taken as a measure of the effect on body water. A similar procedure was
arried out with metamorphosing toads and is described below.

Second, in an attempt to determine how far this effect was due to direct action
f the extract on the kidney, tadpoles were stained with phenol red and the rate of
limination of this dye compared in untreated and treated animals.

The results of the two sets of experiments are correlated in the discussion.

EXPERIMENTS ON WEIGHT CHANGE

The weighing of tadpoles at regular intervals with any degree of accurac presented some difficulty, especially as the earlier stages (*hindlimbs two- or three jointed*) lost up to 30 % of their initial weight as the result of carrying out th process five times over a 6 hr. period. As, however, no alternative presented itsel experimental animals were controlled by others which were weighed at the sam times, but which received no other treatment. The procedure was as follows: eac animal was dried on a soft linen cloth, transferred to a tared specimen tube containing 5 ml. of water, rapidly weighed on an Oertling aperiodic reflecting balanc and restored, together with the water, to its bowl. In the case of terrestrial form the animals were weighed, after drying, in a dry weighing bottle. The great majorit of the animals survived such treatment and later passed normally through metamorphosis.

The Burroughs Wellcome preparation of the posterior lobe, "Infundin", wa used for most (88/127) of the pituitary injections, but Parke Davis "Pitressin (22/127) and "Pitocin" (17/127) were used in confirmatory experiments. Preliminar tests having shown that tadpoles supported the injection of volumes up to 2 % o their body weight without ascertainable injury, the following doses were used "Infundin", 2 I.U.; "Pitocin", 2 oxytocic I.U.; "Pitressin", 4 pressor I.U.; in ever case per 10 g. animal. These doses produced comparable responses, but the pitoci was more active with postmetamorphic stages. This agrees with the findings o Steggerda & Essex (1934) and Boyd & Brown (1938). The extracts were not diluted Injections were made with a Burroughs Wellcome "Agla" micrometer syringe fitte with a fine needle, which enabled quantities as small as 1·5 mg. to be injected wit accuracy. The syringe was clamped to a stand and the animal manipulated so tha the tip of the needle entered at the posterior end of the body, low on the side, an passed a short distance obliquely forward just within the body wall, avoiding injur to the viscera. The injection was then made into the body cavity. The room an water temperatures were between 17 and 20° C. in all experiments.

The experimental procedure was standardized as follows: eighteen numbere tongue glasses, each containing 40 ml. of tapwater for the aquatic stages, or 10 ml for terrestrial animals so that the latter were not drowned, were set out in thre groups of six. These groups were to receive animals, (1) injected with posterio lobe, (2) controls injected with the same volume of 0·5 % NaCl in London tapwater and (3) controls weighed only. Sets of three tadpoles of similar size and metamorphi stage were selected from stock, weighed individually, the time being noted, injecte if destined for groups (1) or (2) and put singly into tongue glasses, one animal t each of the three different groups. This was done six times so that each tongue glas received one animal. After the lapse of 1½ or 2 hr., the tadpoles were weighed agai and so on at equal intervals during the 6 or 8 hr. following the initial weighing The groups were made up of tadpoles of different developmental stages when thes could be obtained simultaneously from stock. In this way, tadpoles treated wit posterior lobe were controlled against saline-injected and "weighed only" animals nd against others at different stages of metamorphosis. Animals of groups (2) and 3) lost similar percentages of their weight in the same period so that this effect can e attributed to handling and to the progressive evacuation of the gut, rather than the injections. Stock tadpoles were observed to feed continuously during the ay, especially in the early stages, but no food was supplied during the experiments.

able I. *Showing the response to injection of extracts of the posterior lobe of the ituitary, as measured by change in weight, of developmental stages of the toad. The nimals were given access to water*

Stage of metamorphosis	No. of animals injected with posterior pituitary extract	No. of animals showing gain in weight after injection	No. of animals showing loss of weight after injection
Hindlimbs three-jointed and earlier stages	63	—	63
Anal canal piece resorbed	6	—	6
Three-limbed	12	2 (both with medium tails)	10
Four-limbed:			
Long tails (> 10 mm. long)	5	—	5
Medium tails (5–9 mm. long)	5	2 (< 4 %)	3
Short tails (< 5 mm. long)	8	4 (< 5 %)	4
Knob tail	8	7 (< 10 %)	1
Dark toads	5	5	—
Yellow toads	15	15 (> 10 %)	—

The results of these investigations are summarized in Table I and typical curves individuals are shown in Fig. 1. It will be seen from the table that posterior be injections have no effect on the body water of the developing toad before tail sorption begins. On the other hand, when the tail is almost completely absorbed *nob-tail*), the majority of the animals show an increase, although not pronounced, water content, whereas later, when the tail has diminished to a small tubercle, response is invariable and of greater magnitude. Thus as development proceeds, gradual increase of sensitivity to posterior lobe extracts is apparent, both in the equency with which water retention is induced, and in degree. The full response not obtained until the skin ceases to be a dark greenish brown (*dark toads*) and sumes the adult colour (*yellow toads*). The period when the positive reaction adually develops appears to coincide with the progress of tail absorption and to egin when this process has been under way for some time, i.e. in *medium* (5–9 *mm. ng*) *tailed* and *short* (< 5 *mm. long*) *tailed* stages. Perfect correlation between the ysiological reaction and external form was not obtained, nor is it to be expected nce it is unlikely that the processes are causally connected. Where a response was tained, this reached its maximum between $1\frac{1}{2}$ and 3 hr. after injection, and the fect subsided within 6–8 hr., as had been found to be the case in adult toads. The ck of response of the earlier stages cannot, therefore, be attributed to the shortness the experiments.

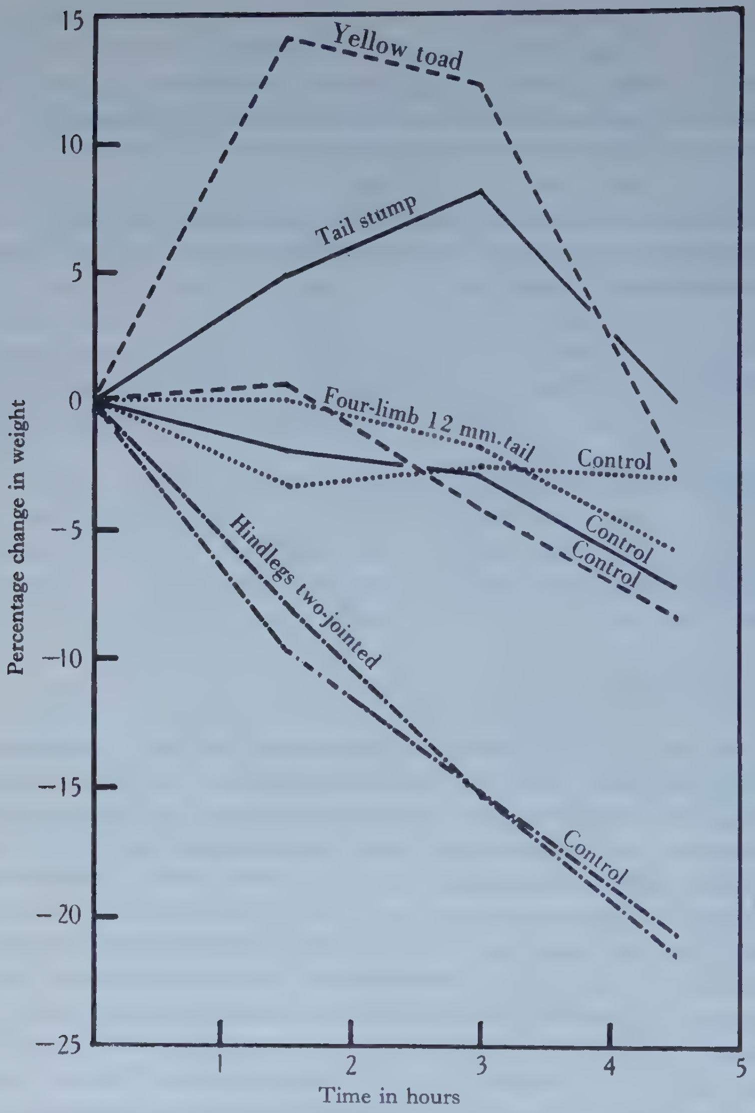

Fig. 1. Percentage weight change of representative animals of four stages in the development of th toad following injection with "Infundin" (2·0 I.U. per 10 g. body weight) or with 0·5 % salin (Controls) and allowed access to water. The same type of line is used both for the "Infundin"-treate animal and its control.

EXPERIMENTS WITH PHENOL RED

The changes in weight following injection with pituitary extracts give n information as to the nature of the mechanism involved in causing the increase water content of the body. This response in Amphibia may possibly be due to tw factors, (*a*) an increased water uptake through the skin, and (*b*) a possible mechanis preventing loss of water through the kidneys. Experiments were undertaken t elucidate this problem.

When tadpoles are immersed in an 0·1 % solution of phenol red for from 12 to 15 hr., they become deeply stained with the dye. When returned to clean water, they gradually give out the dye, at first rapidly and later more slowly until at the end of about 120 hr. it is completely eliminated from their bodies. The animals show no ill effects from this treatment.

The amount of dye given off by the tadpoles in any period could be estimated by comparing the depth of colour of the water in which they had been kept with that of a series of dilutions of the original solution of phenol red. The colour in all cases was brought to that beyond the acid range of the dye by the addition of a few drops of acetic acid. This inversion of the normal procedure used in previous studies on renal activity was necessitated by the cloudiness caused in the water by the addition of alkali. Standard dilutions of the dye and test solutions were compared in Hehner tubes which gave columns about 350 mm. long with 50 ml. water, the tubes being illuminated from below. Many dilutions were made so that the test solution never differed from one standard by more than 30 %.

Preliminary tests were made with ten animals which were immersed in 200 ml. of 0·1 % phenol red (B.D.H. indicator) for 15 hr. and then washed in running water for 15 min. They were then divided into groups of five and each group was placed in 50 ml. of tap water. At intervals this water was poured off through a coarse sieve and collected, and the animals were given a further 50 ml. of clean water.

It was found that in similar groups the amount of phenol red lost by one group might be as much as twice that lost by the other during the first hour, but that during longer periods the maximum loss never exceeded 1·4 times the minimum. The discrepancies were greater in younger than in later forms because dye was retained within the opercular cavity. The method is thus not wholly satisfactory but was considered suitable for preliminary experiments.

Since trial experiments showed that immersion of animals in water containing extracts of posterior pituitary induced changes of weight similar to those following injection, it was decided to use immersion rather than injection as the method of administration to tadpoles in the phenol red experiments. Thus excessive handling of the dyed animals was avoided.

To test the influence of posterior lobe extracts, animals were treated as described above. Two groups of similar tadpoles were placed in clean tapwater after immersion in phenol red solution overnight, followed by washing. At the end of the first hour or so, one group was transferred to a similar measured quantity of tap water to which posterior lobe extract (1·0 I.U. per 10 ml.) had been added. After about 4 hr., this solution was replaced by clean water, the water in the control group being changed as usual. Clean water was again substituted at longer intervals up to about 60 hr., and the concentration of phenol red in the discarded water estimated both before and after treatment with extract. It was found necessary to halve the quantity of water for terrestrial forms and to place a sheet of gauze about 2 mm. above the surface of the liquid so that they remained immersed but could breathe air.

Such experiments were made on the following developmental stages: (1) up to *three-jointed hindlimbs*, five experiments on groups of five animals; (2) *anal canal piece* *resorbed*, three on groups of five; (3) *long tails*, three on groups of five; (4) *knob-tails*, three on groups of five; and (5) *yellow toads*, four on groups of four; with a similar number of controls in each case. It was necessary to omit the *dark toad* stage, as its duration was usually less than that of one of the experiments. This period was not lengthened artificially by conducting the experiments at lower temperatures, since the skin of frogs has a very high temperature coefficient of permeability (Overton, 1904; Hevesy *et al.* 1935) and the water loss of untreated frogs is reduced to one-sixth of the normal at 4° C. (Boyd & Whyte, 1938).

The results of three experiments are shown in Fig. 2. Of the stages investigated, only the *yellow toads* showed a pronounced suppression of phenol red elimination when treated with posterior lobe extract. In none of the four groups was this suppression quite complete, although invariably marked compared with the controls, partly no doubt owing to the time elapsing before the extract penetrated into the animals. There was never any compensatory extra loss of phenol red in the succeeding period, both controls and treated animals losing about the same amount of dye. If it be assumed that the degree of retention of the dye is a measure of the suppression of the secretion of urine by the animal, since the weighing experiments show that water uptake is certainly not reduced, then it may be concluded that the effector mechanism for this action of posterior lobe extracts comes into play only when metamorphosis is quite complete. This is in agreement with the finding that, following injection of extracts, *yellow toads* exhibit a 50 % greater rise in weight than do the other sensitive forms and suggests that while the response of earlier stages is at least predominantly due to increased water uptake (cf. behaviour of *knob-tails* in Figs. 1 and 2), that of the metamorphosed animal is due both to increased transfer of water through the skin and to diuresis inhibition.

DISCUSSION

The fact emerges from the experiments described above that in the duration of the response induced by injections of posterior pituitary extract, the newly metamorphosed *Bufo b. bufo* behaves like all other Salientia that have been investigated. Where the behaviour of two or more species has been compared simultaneously, it has been shown that while the time relationships remain the same (Boyd & Brown, 1938), the amplitude of the weight changes varies from species to species. The more terrestrial toads *B. arenarum* (Novelli, 1933) and *B. americanus* (Steggerda, 1937) show a greater increase in weight than do frogs. There does, then, appear to be some relationship between the degree of response and the danger of desiccation. *Bufo b. bufo* shows a percentage increase in weight which falls within the range found in previous work.

It thus seems justified to compare the behaviour of the English toad with that of the various different species of frog used by other workers.

The exact mechanism of the response is not yet solved in spite of the study that has been devoted to it. Bělehrádek & Huxley (1927) suggest that the effect of posterior pituitary extracts on water regulation in amphibia involves (1) an action on the water intake, i.e. on the permeability of the skin, (2) an action on the powers

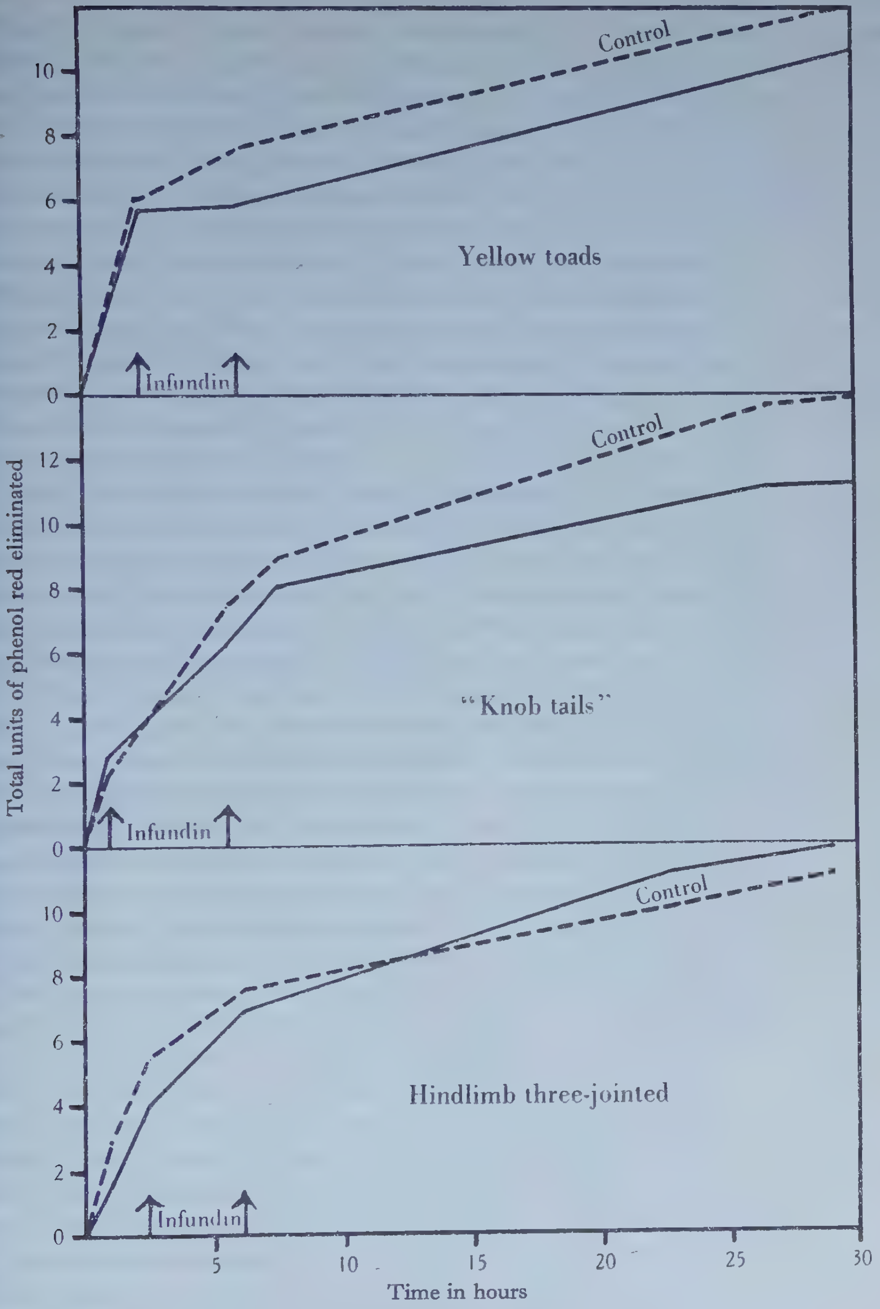

Fig. 2. Elimination of phenol red by groups of toads at different developmental stages. Each point epresents the total amount of phenol red eliminated by a group from the beginning of the experiment. The experimental animals, represented by unbroken lines, were placed in dilute infundin (1 I.U. per o ml. water) for the period indicated by the arrows. The control animals were simultaneously placed n water.

of imbibition of the tissues, and (3) an action on water excretion, i.e. on kidney activity. The brief response of the water-regulating mechanism to a single injection of Infundin is scarcely comparable to the action, which may be due to different agencies, of posterior lobe extracts in retarding metamorphosis (Spaul, 1925) nor are the profound metabolic disturbances associated with this process considered relevant. If the weighing experiments are a measure solely of the increased water uptake of the skin, and the kidneys are unaffected, the frog must be almost unique in normally working its kidneys so near the upper limit of their capacity that they are unable to cope with the extra water inflow. Normally, *Rana pipiens* excretes about one-third of its weight of water per day (Adolph, 1933), but Boyd & Whyte (1939) found that frogs could take in and lose up to 20 % of their weight in 3 hr. The accumulation of water in a frog injected with pituitrin is at the rate of about 160 % of its weight per day, i.e. the rate of water uptake is apparently increased to five times the normal if Adolph's low figure be taken. Frogs used by Richards (1938) had a normal rate of urine formation of 2·5 ml./hr., although it could be calculated from the glomerular number and the maximum rate of filtration through a single glomerulus that the kidneys might form glomerular filtrates at rates up to 16 ml./hr., over 6 times as fast. Again, evidence from injection experiments with posterior pituitary shows that the kidneys can deal with a great deal more water than Adolph's figure suggests, since after the peak increase in weight, animals so treated do in fact lose more than 20 % of their weight as water in about 3 hr. Increased water uptake by the skin cannot then be the sole cause of the rise in weight. Nevertheless Novelli (1936), by experiments with skin pouches in which the circulation was kept intact, showed that it is an important factor.

The powers of imbibition of the tissues are affected by injections of "Pitressin" into the intact animal and the hydration of the muscles is considerably increased (Steggerda, 1931) and Rey (1935) showed that there was an increased hydration of the blood as well as of the tissues but that also a decreased kidney output was responsible for the gain in weight. In recent years the importance of this last factor has been shown to be greater than was at first believed. Rey (1935) and Boyd & Whyte (1939) found injected water to be retained after treatment with pituitrin and concluded that extracts of the posterior pituitary contained a substance which rendered the kidneys unresponsive to increased hydration of the blood.

Thus all three factors, namely, increased permeability of the skin, imbibition of water by the tissues and a failure of the kidney to respond to changes in the internal medium brought about by these, are concerned in the response of adult Salientia to injections of pituitrin. It may be concluded that the same factors are at work but in differing degrees, in the response of the developing toad. Since, however, until the later stages these animals are solely aquatic, water exchange in both directions through the alimentary canal may also be important. We know that during metamorphosis the urodele skin undergoes profound morphological changes (Wilder, 1925; Reis, 1930), so it may be concluded that the skin of the toad undergoes a similar alteration in structure, although we have no data on the nature of these changes nor at what stage they take place. It is likely that the tissues can imbibe water as in the adult. From the results of the weighing and phenol-red experiments described above, the conclusion may tentatively be drawn that the early response of the developing toad from the *four-limb*, *medium tail* stage onwards is due to the skin becoming sensitive to the influence of the posterior pituitary. Phenol red injected into the blood stream of frogs is eliminated partly by glomerular filtration but mainly by secretion from the tubules (Richards & Walker, 1930; Smith, 1937; Richards, 1938), so that these experiments merely show that pituitrin had some effect on the kidney without giving any information as to whether that effect is due to a suppression of glomerular filtration or to an increased resorption of water. Either or both of these factors would reduce the elimination of phenol red by suppressing the secretion of urine. Actually, according to Adolph (1936), "Infundin" reduces glomerular blood flow and this is correlated in degree with the rate of urine formation. Blount's (1936) evidence also indicates that the glomerulus is affected. The "pressor" action of posterior pituitary extracts does not appear to influence water retention by the kidneys.

The data presented imply that suppression of urine production can only be brought about by pituitrin at the very end of metamorphosis but the experiments allowed of observations of only a general nature. If the number of nephrons progressively increases from about the *hind-limb-bud* stage onwards as in the frog (Gray, 1930), more refined experimentation may make it possible to show that this response gradually develops as more nephrons appear.

The factors involved in the action of extracts of the posterior pituitary on the body water of urodeles have not yet been investigated. Steggerda (1937) showed that *Necturus maculatus* increased slightly in weight after injection with pituitrin and Bělehrádek & Huxley (1927) produced evidence that metamorphosed and larval axolotls both gave a limited response, but the time relations in the latters' experiments were different from those of later work and the larvae failed to survive the treatment. More work is needed before one can decide whether changes in the mechanism of water regulation, similar to those described above for *Bufo bufo bufo*, occur during metamorphosis in urodeles and whether the response to posterior lobe extracts is characteristic of primarily terrestrial vertebrates or not.

SUMMARY

1. The effects of extracts of the posterior lobe of the pituitary on the body water of the developmental stages of the toad, *Bufo bufo bufo* (L.) have been investigated by injecting extracts and weighing the animals at short intervals afterwards.

2. No response was shown until marked absorption of the tail had occurred. From this stage onwards it became progressively established and gradually increased in magnitude. The adult Salientian type of response was only elicited at the very end of metamorphosis.

3. By staining tadpoles with phenol red and measuring the rate of elimination of this dye, it has been shown that the action of the extracts is not antidiuretic except in completely metamorphosed animals.

4. It is provisionally concluded that the extracts have a double effect, first to increase the rate of water uptake and second, to diminish water loss. During the life-history of the toad, the first response is established when tail absorption is progressing rapidly, i.e. at a comparatively early stage, whereas the latter only comes into play at the very end of metamorphosis.

5. Data at present available suggest that an influence of the posterior pituitary on body water is characteristic of terrestrial but not of primarily aquatic vertebrates.

My sincere thanks are due to Prof. D. M. S. Watson, F.R.S., for his interest during the experiments and to Prof. E. A. Spaul for his constructive criticisms of this paper and for extending to me the hospitality of his Department, so enabling this work to be completed. The "Pitocin" and "Pitressin" were kindly supplied by Messrs Parke, Davis and Co.

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THE EXPERIMENTAL MODIFICATION OF THE OESTROUS CYCLE IN THE FERRET BY DIFFERENT INTENSITIES OF LIGHT IRRADIATION AND OTHER METHODS

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(With Plate I)

The original discovery by Bissonette (1932) that the recurrence of oestrus in the ferret was greatly accelerated by light was corroborated and extended by Marshall & Bowden (1934, 1936), who found that ultra-violet light had a more long-continued effect than rays from the visible part of the spectrum, but that rays of long wavelengths caused no acceleration. The experiments described in this paper were upon (1) the effects of different degrees of light intensity as obtained by placing the cages at different distances from the electric lamp, (2) the effect of ultra-violet light on the duration of oestrus, (3) the effect of feeding vitamin D to anoestrous animals, and (4) the effects of copulation as shown by the discontinuance of oestrus and the subsequently observed state of the ovaries and other reproductive organs. Under present circumstances it has not been possible to continue the experiments, but the results, although not so complete as originally hoped for, seem sufficiently conclusive to make their publication desirable.

INTENSITY OF RADIATION

The general arrangement of the cages and the source of light were the same as in the original experiments (Marshall & Bowden, 1934). As before, the source of illumination was a large Sollux filament lamp of 1000 W., and the backs of the cages were formed by sloping glass mirrors. The illuminated cages were placed at distances of from 1 to 22 ft. away from the lamp. The light was automatically turned on about sunset and turned off about 8 hr. afterwards, the precise times varying according to the season of the year, that is to say, the light was turned on at times varying from about 4 p.m. to 9 p.m. and turned off at times varying from about 12.30 a.m. to 5 a.m. The condition of oestrus was in all cases clearly demonstrated by the increase in the size of the vulva which at its maximum is enormous.

In the first two years (1935–6 and 1936–7) the experiments were almost completely a failure, owing to mortality among the ferrets from distemper and other causes which resulted among other things in delay in starting. The results, such as they were, seemed to indicate that there was little difference in the times of onset of oestrus in ferrets placed at short distances (1–6 ft.) from the light. The results were, however, partly vitiated by the fact that artificial irradiation having begun late (not until December) the time when the ferrets came on heat (in each case in January) were nearer the normal sexual season. The ferrets placed at longer distances from the light unfortunately died in the course of the experiment and without coming on heat.

The results of the experiments for the two following years are given in Tables I and II. Experimental irradiation was begun on 9 October in 1937 and on 10 October in 1938. It was discontinued on 14 March in both 1938 and 1939. The tables show

Table I. 1937–8

No. of ferret	Distance from lamp ft.	Started coming on heat	Started going off heat	Started coming on heat 2nd time	Notes
51	1	10 Nov.	—	—	Killed in poor health, but still on heat 9 Feb.
52	1	17 Nov.	23 Feb.	29 June	Put with male 9 Feb. Killed 21 July when still almost fully on heat 2nd time
53	2	1 Dec.	15 Feb.	22 June	Put with male 9 Feb. Started going off heat 2nd time 3 Aug. Died
54	2	24 Nov.	15 Feb.	15 June	Started going off heat 3 Aug. Died
55	4	8 Dec.	16 Mar.	—	Killed 17 Apr.
56	4	5 Jan.	9 Mar.	—	Killed 17 Apr.
57	6	22 Dec.	2 Mar.	29 June	Going off heat 24 Aug.
58	6	15 Dec.	9 Mar.	22 June	Going off heat 10 Aug.
59	14	22 Dec.	23 Mar.	22 June	Going off heat 14 Sept. Died
60	14	26 Jan.	13 Apr.	8 June	Going off heat 14 Sept. Died
61	22	—	—	—	In very poor health, thin. Killed 2 Feb.
62	22	—	—	—	In very poor health, thin. Killed 2 Feb.

Table II. 1938–9

No. of ferret	Distance from lamp ft.	Started coming on heat	Started going off heat	Started coming on heat 2nd time	Notes
67	1	9 Nov.	—	—	On heat 18 weeks until killed 15 Mar.
68	1	16 Nov.	26 Apr.	28 June	On heat the first time for 22 weeks. Copulated 4 Aug. Killed 16 Aug. when nearly off heat
69	2	9 Nov.	—	—	On heat 18 weeks until killed 15 Mar.
70	2	16 Nov.	12 Apr.	5 July	On heat the first time for 20 weeks. On heat the 2nd time for only 10 weeks (off heat 13 Sept.)
71	4	16 Nov.	—	—	On heat 17 weeks until killed 15 Mar.
72	4	23 Nov.	19 Apr.	12 July	On heat the first time for 20 weeks. On heat the 2nd time for only 9 weeks (off heat 13 Sept.)
73	6	30 Nov.	—	—	On heat 15 weeks until killed 15 Mar.
74	6	30 Nov.	22 Feb.	5 July	On heat the first time for 11 weeks. On heat the 2nd time for 10 weeks
75	14	1 Mar.	29 Mar.	21 June	Died 26 June
76	14	22 Feb.	—	—	On heat 24 weeks until 9 Aug. when it copulated and then went off heat. Killed 16 Aug.
77	22	1 Feb.	12 Apr.	28 June	On heat the first time for 10 weeks. On heat the 2nd time 9 weeks until 3 Aug. when it copulated. Killed 16 Aug. off heat
78	22	28 Dec.	—	—	On heat 11 weeks until killed 15 Mar.

that the acceleration in the times of oestrus was very roughly in relation to the intensity of the irradiation, as shown by the distance of the ferrets from the lamp, but that there was considerable individual variation which may have been due to differences in the ages of the animals or other unknown individual factors.

ULTRA-VIOLET IRRADIATION

In 1935 two ferrets were submitted to unfiltered ultra-violet irradiation at close quarters (about 1 ft. distance from the bulb). The bulb employed was an "Hanovia" Utility Model Analytic Lamp fitted with a "tilt type" mercury vapour quartz burner, supplied for operation on 200 V. alternating current mains. The radiation began on 2 December 1935 and ended on 16 March 1936. In each case the ferrets started coming on heat about 26 February and remained fully oestrous until 12 August 1936, when they began to go off heat. The period of full oestrus was 22 weeks in each case.

The ultra-violet irradiation experiments for 1936–7 concerned four female and two male ferrets. The irradiation started on 5 December and ended on 14 March. The bulb used was as before, the ferrets being placed horizontally to it, except where otherwise stated, and 1 ft. away.

Of the four female ferrets two came on heat on 13 and 20 January, but they died from unknown causes in March and early April.

Of the other two, one (no. 36) was put with a male, which received ultra-violet treatment, on 21 July but was not seen to copulate though it may have done so. It was still fully on heat on 28 July. It was then put with a male that had been submitted to light irradiation treatment and was believed to have copulated but he completion of the act was not seen. It went off heat on 14 August and was hought to have probably become pregnant; this, however, was a mistake as no young were born. It did not come on heat again. This ferret was 18 in. from the amp which was placed above it and in this instance the rays were filtered by a copper sulphate glass plate.

The other female (no. 44) was put with another male which had received full ultra-violet irradiation on 21 July. It was taken out on 28 July after having probably copulated though copulation was not observed. It was going off heat on 28 July and was completely off on 11 August. Nevertheless, it did not become pregnant as no young were born. It remained off heat until 1938 when it came on at the normal ime on 9 March.

The two male ferrets were used to test the effect of ultra-violet irradiation on he descent of the testes which usually occurs in the spring shortly before breeding. n each case the testes were observed to have descended as early as 16 December. The testes remained descended until 29 September when they once more ascended n the inguinal canals.

In 1937–8 six females were submitted to ultra-violet irradiation as before except hat the bulb was renovated. This may have resulted in the treatment being too orceful as all the animals became very thin and four died in February, after first coming on heat. The irradiation began on 9 October and ceased for the remaining ferrets on 2 March. One of these came on heat on 23 February in spite of being in very poor condition. It was exceptional in going off heat on 30 March. It came on heat again, however, on 29 June and remained so until 14 September. The remaining ferret came on heat on 25 January and remained in that condition continuously until 5 October, a period of 34 weeks. This ferret, in view of its very poor condition, was taken away from the lamp for 1 week (17–24 November). It was given three drops of "radiostoleum" (a preparation containing vitamins A and D) every other day from 8 December to 9 February, and this treatment improved it. The two ferrets which survived to the autumn were placed horizontally at 12 in. from the lamp.

In 1938–9 the experiments were again partly vitiated by the ferrets getting into an unusually poor condition. The irradiation was started on 10 October and ceased on 14 March. It is possible that the irradiation was again too intense. The results are shown in Table III.

Table III. *Ultra-violet irradiation*, 1938–9

No. of ferret	Started coming on heat	Started going off heat	Started coming on heat 2nd time	Remarks
63	15 Feb.	10 May	June 28	Remained on heat until disposed of Oct. 1939. Became very thin at end of Dec. 1938 but improved in the spring
64	8 Mar.	20 Sept.	—	Thin. Very late in coming on heat but remained oestrous for 25 weeks
57	23 Nov.	—	—	Behaved like most ultra-violet ferrets in previous years, remaining on heat for 38 weeks, that is, until killed on 16 Aug.
58	9 Nov.	14 Dec.	18 Jan.	Remained on heat the second time for 30 weeks, being killed on 16 Aug.
65	16 Nov.	22 Feb.	5 July	Remained on heat until sold in October
66	9 Nov.	14 June	9 Aug.	Remained on heat until sold in October

Notes

Nos. 63 and 64 were young virgin ferrets.

Nos. 57 and 58 were used in the intensity experiments (1937–8).

Nos. 65 and 66 were used in the vitamin D experiments (1937–8 described below).

Nos. 57, 58, 63 and 64 were at a distance of 12 in. from the lamp and placed horizontally. Nos. 65 and 66 were 18 in. from the lamp and placed vertically.

It will be seen that the tendency for ferrets treated with ultra-violet rays to remain on heat for an exceptionally long time was maintained, but that certain individuals in the last two years being in bad condition did not behave like the others submitted to this treatment.

EFFECTS OF VITAMIN D

In view of the action of ultra-violet irradiation in causing a long-continued condition of oestrus, the suggestion presented itself that the effects might be due to the production of vitamin D. Such a conclusion was, however, unlikely in view of the general evidence that the incidence of oestrus in animals is regulated by exteroceptive stimuli acting through the nervous system and pituitary. Nevertheless it was thought that the possible action of vitamin D as a factor ought not to be ruled out in the absence of experimentation. Accordingly, four ferrets were treated with the commercial preparation "radiostoleum" (British Drug Houses, Ltd.), which contains a standardized quantity of crystalline vitamin D (3000 I.U. per g.), as well as a standardized solution of vitamin A (15,000 I.U. per g.). Four drops were added daily to the usual bread and milk diet which was daily eaten. The experiments were begun on 27 October 1937, and continued until 15 February 1938. None of the ferrets came on heat during this period. They actually came into oestrus about 16, 23 and 30 March, and 13 April, which dates are approximately at the time when the normal sexual season commences. The feeding of radiostoleum, however, caused all the ferrets to become abnormally fat, far more so in fact than with any other ferrets that we have seen. This result was not anticipated, so we did not take records of the weights, but nevertheless there can be no doubt that it was significant. After ceasing to feed radiostoleum the ferrets gradually became thinner and returned to their normal appearance.

EFFECTS OF IRRADIATION UPON THE OVARIES AND UTERUS

Since the experiments were carried out primarily to determine the influence of irradiation upon the incidence of oestrus and its duration the ferrets were usually kept alive until they had passed back into a condition of sexual rest. Consequently material was not usually available for the study of the reproductive organs of the treated animals. In some cases, however, the ferrets were killed during the oestrous periods and their organs were preserved and subsequently investigated. It had been noted that with those ferrets which were put with males (nos. 36 and 44) and were thought to have copulated, the effect of being with the male was to terminate the oestrous condition in a short time. None of these animals, however, became pregnant or palpably pseudo-pregnant, so the possibility presented itself that the Graafian follicles had become cystic as a result of the prolonged oestrus and consequently had not discharged their ova. The condition of the ovaries described below lends no support to this view.

Ferret no. 67 mentioned above in the "intensity series" (1938–9) was at a distance of 1 ft. from the lamp and was continuously on heat for 18 weeks before being killed on 16 March. The ovaries were entirely normal. Large protruding ollicles could be seen on the surface with the naked eye. Sections showed that ome of the follicles were mature and normal and there were other smaller ones at ll stages. No recent corpora lutea were observed and no atrophic follicles. There was no evidence that follicles had become ripe in batches and subsequently degenerated. There was a great abundance of typical interstitial cells but no cells hat could certainly be said to be old luteal cells.

The uterus as seen in section was in a general way similar to that of the normal erret which had been for a long time on heat described in an earlier paper (Hammond & Marshall, 1930). There was some congestion and the glands showed evidence f prolonged activity. The epithelium lining the cavity was normal and the cells did not present the appearance of a plasmodial condition such as is shown by ferrets which become pseudo-pregnant.

Ferret no. 78 in the same series was similar but it had been at a distance of 22 ft. from the lamp (the long distance extreme). It was on heat for 11 weeks before being killed on 16 March. There were numerous large follicles and many smaller ones. No corpora lutea were observed but there was one apparently collapsed follicle which was degenerate; it contained a persistent ovum. Interstitial tissue was abundant as in no. 67.

The uterus as seen in section was very similar to that of no. 67; there was some congestion and evidence of glandular activity was also seen.

Ferret no. 57 was in the ultra-violet series (1938–9). It had been on heat continuously for 38 weeks before being killed on 16 August. The ovaries contained very large ripe follicles but no cystic ones and no corpora lutea. There were, however, three old atrophic follicles. These had their cavities filled up in the usual way with a rather loose ingrowth of tissue from the walls but no remains of ova were seen. It is possible that these represented follicles that had ripened and then degenerated, being replaced by other follicles which in time enlarged in the manner described for the rabbit (Smelser *et al.* 1934). Interstitial cells of the usual type were fairly numerous in the ovarian stroma.

The uterus showed large active glands. The surface ones were not pitted and there was nothing to suggest a condition of pseudo-pregnancy. The epithelium was continuous and not plasmodial.

Ferret no. 58 was also in the ultra-violet series. As recorded above, it was exceptional in not remaining on heat the first time for more than 5 weeks. This behaviour suggested that it might possibly have ovulated or that some of the tissue had become lutealized. It came on heat again in January when it remained oestrous until killed on 16 August. The ovaries showed a lot of old luteal tissue. Some of this was definitely surrounded by walls so as to present the appearance of old corpora lutea. The luteal cells were similar to those found in ferrets' corpora lutea at the end of pregnancy or just after parturition. (Compare the ferret a fortnight after pseudo-pregnancy in Hammond & Marshall's series (1930).) The ferret had never been with a male. It is possible, therefore, that the luteal tissue had been formed as a postponed result from the former treatment with ultra-violet light acting on the anterior pituitary, the normal cyclic changes which depend upon that organ having been deranged.

The uterus of no. 58 as seen in section showed a somewhat abnormal appearance. There were numerous small glands as well as some very large glands. The appearances were such as to suggest a normal ferret coming on heat again a little while after pregnancy or pseudo-pregnancy. (Cf. no. 15 in Hammond & Marshall's series (1930), a ferret 15 days after parturition with young removed, but in no. 58 in the present series some of the glands were still larger.) This ferret, however, had been on heat continuously from mid-January to mid-August. It was possible that the ultra-violet irradiation had caused the pituitary to secrete some amount of the lutealizing hormone along with the follicle-stimulating hormone, but that the estrogenic effect was dominant the greater part of the time. The fact that this erret went off heat for a time in December may perhaps be similarly explained. he uterine epithelial cells were continuous and not plasmodial.

DISCUSSION

The effect of artificial irradiation in accelerating the oestrous cycle in various pecies of animals, both vertebrate and invertebrate, has been well summarized in rticles by Bissonette (1936 *a*, *b*) and Rowan (1938) and need not be further described ere. The evidence relating to the nervous paths of transmission of impulses to the rain and thence to the anterior pituitary is given in a paper by Le Gros Clark *et al.* 1938) which adds considerably to our knowledge. The experiments recorded above uggest that exteroceptive stimulation by ultra-violet irradiation may result not nly in the release of the follicle-stimulating hormone but also in that of the lutealizing ormone from the pituitary, the former, however, always being dominant. As is ell known, ferrets only ovulate after coition (Marshall, 1904) and the existence of ell-defined luteal tissue in the ovary of a ferret which had never been with a male ay probably have been due to the release of the lutealizing hormone consequent pon ultra-violet irradiation. It is to be noted that a similar result may follow in ome animals after the injection of anterior pituitary extracts as described by Hill Parkes (1930).

SUMMARY

With female ferrets subjected to different degrees of intensity of light irradiation s measured by placing them at different distances from a 1000 W. lamp, the cceleration of the oestrous cycle, generally speaking, was correlated with the degree f intensity. There were, however, individual exceptions. The distances between ne ferrets and the lamp varied from 1 to 22 ft.

Female ferrets subjected to ultra-violet irradiation usually remained on heat ntil much later in the year than those submitted to light irradiation. With male errets ultra-violet irradiation caused descent of the testes in December and the estes remained descended until the autumn.

Irradiated female ferrets (both with light and ultra-violet rays) went off heat nortly after being put with males and probably as a result of copulation (which, owever, was not always observed), but pregnancy did not supervene. This may ave been due to the animals not being in a state for ovulation.

Feeding vitamin D to anoestrous ferrets did not result in accelerating the cycle, ne ferrets not coming on heat until the normal time. The treatment, however, esulted in their becoming abnormally fat. Vitamin D was given in a commercial reparation which also contained vitamin A.

Sections through the ovaries of irradiated oestrous ferrets showed large ripe ollicles and other follicles in varying degrees of development. Some apparently nruptured degenerate follicles were also seen. There were no cystic follicles in any

of the ferrets' ovaries observed. Typical interstitial cells were present usually i great abundance.

In a ferret that had been submitted to ultra-violet irradiation the ovaries contained a quantity of old luteal cells, the presence of which suggested that some o the follicles had become lutealized without rupturing.

The uterus in three of the irradiated ferrets was somewhat congested and showe signs of glandular activity, being, generally speaking, similar to that of norma ferrets which have been on heat a long time. The uterus of a female ferret, in whic the ovaries contained old luteal cells, presented the appearance of one belongin to an animal that was coming on heat again after pregnancy or pseudo-pregnancy

I desire to express my indebtedness to Dr F. P. Bowden for his valuable assistanc at the beginning of these experiments and my regret that his subsequent absenc from Cambridge rendered his collaboration no longer possible. I wish also to than Mr J. Pike for his help in superintending the experiments. Furthermore, I desir to express my obligations to the firm of Hanovia Ltd. for the information whic they willingly supplied.

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EXPLANATION OF PLATE I

Fig. 1. Section through ovary of ferret no. 78 after additional prolonged irradiation (see text) showing portion of normal enlarged follicle with ovum and very numerous interstitial cells.

Fig. 2. Section through ovary of ferret no. 57 after ultra-violet irradiation (see text), showing portio of normal enlarged follicle with ovum and two old atrophic follicles.

Fig. 3. Section through ovary of ferret no. 58 after ultra-violet irradiation (see text), showing portio of follicle and a quantity of typical luteal tissue, some contained within old follicles.

Fig. 4. Transverse section through horn of uterus of ferret no. 58, showing enlarged glands and othe evidence of a pro-oestrous or oestrous condition (see text).

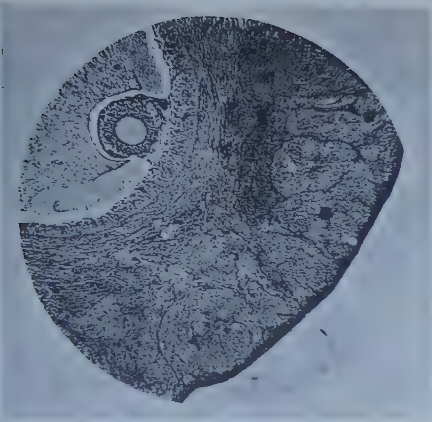

Fig. 1.

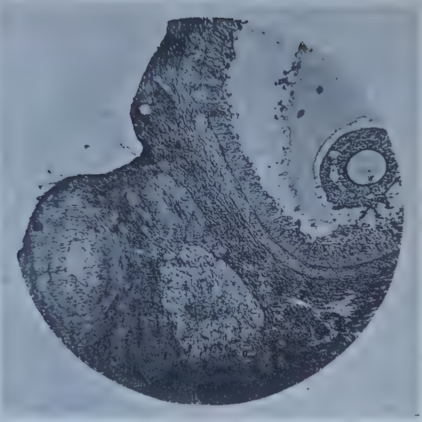

Fig. 2.

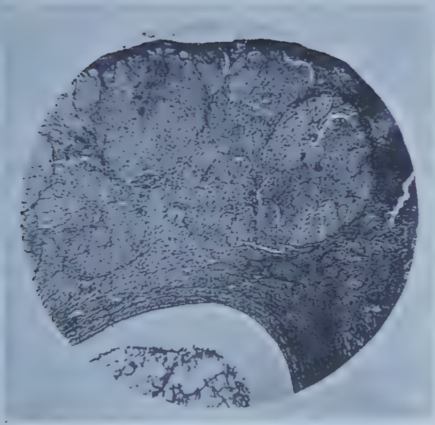

Fig. 3.

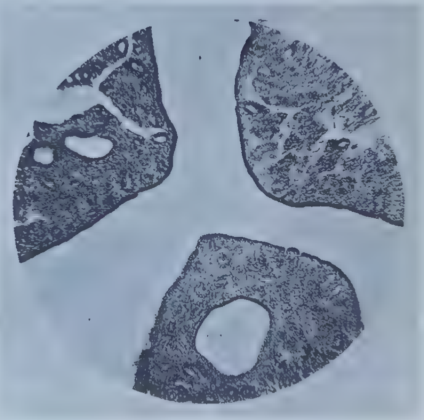

Fig. 4.

MARSHALL—THE EXPERIMENTAL MODIFICATION OF THE OESTROUS CYCLE IN THE FERRET BY DIFFERENT INTENSITIES OF LIGHT IRRADIATION AND OTHER METHODS (pp. 139–146)

NOTE ON THE BIOCHEMISTRY OF EMBRYONIC DETERMINATION IN ECHINODERMS

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PART from the Amphibia, the echinoderms are the class of animals about which ur knowledge of embryonic determination and its biochemical basis has in recent imes made the most important forward strides. This is largely due to the brilliant ork of the Scandinavian school. The fundamental facts of echinoderm experi-nental embryology are now available in a review in the English language by örstadius (1939), while on the biochemical side there are general accounts by unnström (1935*a*) and particularly by Lindahl (1936).

We may mention, however, that two points of radiating influence (whether or ot they should be called organizer centres remains debatable) in the echinoderm mbryo, one at the animal pole, the other at the vegetal. As first proposed by unnström, their action may best be represented in the form of two gradients, one aximal at the animal pole, the other at the vegetal. Normal development to the luteus depends upon the "co-operation" of these two gradients, and many, if not ll, of the anomalies of development may be traced to changes in their intensity. hould one centre or gradient apex be depressed, the development of the embryo eers towards the type "advocated" by the other. Since the vegetal pole forms ndodermal tissues, it will, if circumstances allow it wholly to control development, ring about a "vegetalization" or "endodermization" of the embryo. In this case, resumptive ectoderm is made to form endoderm. Conversely, since the animal ole forms ectodermal tissues, it will, if given a like control of development, bring bout "animalization" or "ectodermization", in which case presumptive endoderm s made to form ectoderm. When the gradients are equally powerful, the normal luteus larva results, with its ectodermal skin, its mesenchymal spicule skeleton, nd its endodermal tripartite gut. When vegetalization occurs, much material other han presumptive endoderm is deviated to form gut, which may hang out from an xo-gastrula, the ectodermal portion being reduced to a thin-walled, irregularly haped, bag, containing deformed spicules. Conversely, when animalization occurs, o gut or skeleton ever forms at all, and the embryo becomes a thick-walled ecto-ermal bag provided with a very excessive complement of cilia and a hypertrophied pical tuft.

The characteristic thing about echinoderm development is that these changes can be brought about, either by morphological methods (such as isolations and various combinations of blastomere groups, or transplantations of the very vegetal micromeres); or by chemical methods (especially ionic changes in the medium); or by a mixture of both. The classical example of a vegetalizing agent is Li′, which brings about exo-gastrulation in its completest form; its action can be intensified by respiratory inhibitors such as KCN and CO. Among the most important animalizing agents are SCN′ (especially when acting before fertilization), the action of which is intensified by respiratory accelerators such as pyocyanin; and lack of SO_4'' ions in the medium. These agents show many phenomena of antagonism and reversibility. They also act in a very regular and predictable way, and as already mentioned, may be combined with purely morphological methods. Such an experiment as that of Hörstadius (1936), in which animal blastomeres were isolated and then treated with Li′ before transplantation, is a case in point. If such isolated animal blastomeres are vegetalized in this way, they will, on introduction into another embryo, act as powerfully in a vegetalizing direction as the micromeres themselves.

A good deal of effort has been devoted by the Scandinavian school to the biochemical explanation of the developmental deviations which chemical treatment will bring about. But a study of the literature shows nevertheless that on the whole no biochemical effects of the lithium ion comparable in magnitude to the exo-gastrulation effect, have been discovered. That there is a lithium-inhibitable fraction of respiration during the cleavage stages is sure, but it is not more than 25 % of the total. In brei experiments (cytolysed eggs), the reduction time of methylene blue in the presence of hexose-monophosphate as donator is considerably retarded by lithium. On these grounds, Lindahl put forward the tentative hypothesis that the metabolism characteristic of the animal pole may be carbohydrate in nature. In the same way lack of SO_4'' ions also inhibits respiratory rate, but the fraction sensitive must be different because the inhibitions sum. Suggesting that the only function which sulphate ions could be likely to serve was that of detoxicating and removing aromatic waste products of protein breakdown, Lindahl framed the hypothesis that the metabolism of the vegetal pole of the egg (and hence reduced, or abolished, or rendered toxic, by animalizing agents) was of protein nature. In support of this, he succeeded in showing the existence of phenol-sulphatases in echinoderm embryos.

In the present note we shall have no more to say about the metabolism of the vegetal pole, but it occurred to us that some further light might be thrown on that of the animal pole by the use of well-known inhibitors of carbohydrate breakdown. A few experiments along this line had already been reported by Runnström (1935 *b*). He exposed the unfertilized eggs of *Arbacia* to $M\ 3 \times 10^{-2}$ iodoacetate for from 2 to 6 hr., after which the small number of survivors which continued development when transferred to normal sea water showed poor development of the stomodaeum and oral arms, though the organization of the pluteus was in other respects ordinary. These effects he interpreted as very slight vegetalization, and hence confirmatory of Lindahl's hypothesis. In $M\ 3 \times 10^{-4}$ bromacetate, Tchakhotin (1938) observed great proliferation of primary mesenchyme, entirely filling the blastocoele cavity; his is very anomalous and cannot be regarded as evidence of vegetalization.

No experiments with *dl*-glyceraldehyde, however, existed. Mendel (1929) and Iendel *et al.* (1931) made the original discovery that this substance inhibits the lycolysis of tumour tissue, mammalian brain tissue, etc., and that the inhibition s maximal at M 10^{-3}. Much the same concentration was found to be maximally ffective on the glucolysis of the chick embryo (Needham & Nowinski, 1937) and he amphibian tadpole (Nowinski, 1939). Needham & Lehmann (1937) were able o demonstrate that the inhibition is due only to the *l*-isomer, and this was confirmed y Mendel *et al.* (1938).

Many other workers (references to some of whom are given in the paper of Jeedham & Lehmann, 1938) have obtained similar inhibitions with glyceraldehyde n other tissues, but it has generally been believed that the processes affected are f the non-phosphorylating type, since such concentrations of *dl*-glyceraldehyde do ot affect the glycogenolysis of muscle extract, for example. If, however, much tronger concentrations are used (from 10^{-2} up to molar), as in the experiments of Idler *et al.* (1937) or Boyland & Boyland (1938); or possibly if at intermediate oncentrations the glyceraldehyde is present in the freshly dissolved, and hence, imeric, form; phosphorylating glycolysis may also be inhibited.

It is well known that phloridzin also inhibits phosphorylating glycolysis Parnas, 1937); it is maximally effective at about M $0{\cdot}5 \times 10^{-2}$.

With this introduction, we may proceed to consider the experimental data we vere able to procure. At Salsbury Cove, Mount Desert Island, Maine, we had an xcellent supply of the sand-dollar *Echinarachnius parma*, and at Wood's Hole, Iass., we used the sea-urchin *Arbacia punctulata*. We shall omit all technical etails concerning the preparation of the material, since these will be found in the ecently published notes of Just (1939). The eggs were placed in the inhibitor olutions (inhibitor dissolved in sea water) as soon as it was ascertained that pproximately 100% fertilization membranes had appeared. From Table I it will e seen that normal development will proceed up to M 5×10^{-4} glyceraldehyde in he case of *Echinarachnius* and Table II shows that the same is true of *Arbacia*. t M 10^{-3} there are abnormalities of development in both cases, but the important oint is that there was never the slightest trace of exo-gastrulation. Whether these bnormalities may be regarded as traces of vegetalization seems open to doubt. At I 10^{-2} development ceases at the morula stage. Table II includes two lots of mbryos which were subjected to the higher glyceraldehyde concentrations before ertilization only, developing subsequently in ordinary sea water. In this way it was ossible to get some development after treatment with M 10^{-3} and even 5×10^{-3}, ut in the latter case development stopped after gastrulation and before the appearnce of any arms or spicules.

The experiments with iodoacetate and phloridzin were less successful. Normal evelopment took place in iodoacetate (see Tables I and II) in concentrations as igh as M 5×10^{-4} but higher than this, even when the treatment was confined to he unfertilized eggs, no development beyond the gastrula stage was ever obtained.

Table I. *Echinarachnius parma*, *T* 14° C.

Exp. 3 and 4:

	20 hr.	32 hr.	44 hr.	54 hr.	68 hr.	
Control	Vigorous blastulae just beginning to gastrulate	Vigorous prismatic gastrulae	Young plutei with spicules forming	Normal plutei	Long-armed plutei	
Glyceraldehyde:						
10^{-2}	Morulae	—	—	—	—	no exo-gastrulae
10^{-3}	As control	As control	Prismatic gastrulae	Gastrulae with long guts but no spicules	Degenerating	
5×10^{-4}	As control	As control	As control	Young plutei	Plutei	
10^{-4}	As control	As control	As control	As control	As control	
10^{-5}	As control	As control	As control	As control	As control	
Iodoacetate:						
3×10^{-2}	Morulae	—	—	—	—	no exo-grastrulae
3×10^{-2}*	As control	No further development	—	—	—	
10^{-2}	Morulae	—	—	—	—	
10^{-3}	Morulae	—	—	—	—	
5×10^{-4}	As control	Mid-gastrulae weakly swimming	No further development	—	—	
10^{-4}	As control	Mid-gastrulae	No further development	—	—	
Phloridzin:						
10^{-2}	Morulae	Early gastrulae	Advanced gastrulae	—	—	no exo-gastrulae
5×10^{-3}	Blastulae motile but unhatched	Mid-gastrulae	No further development	—	—	
10^{-4}	As control	As control	No further development	—	—	
Lithium chloride 0·1 %	Vigorous blastulae, but spherical	Gastrulation inhibited, and many exo-gastrulae	90 % exo-gastrulae	The same	—	

* 4 hr. treatment before fertilization only.

Nevertheless, as Table II shows, this stage was often reached without the slightes sign of exo-gastrulation even at concentrations as high as $M\ 3 \times 10^{-2}$, and since the maximally effective concentration in the case of tissues of vertebrates is $M\ 10^{-3}$, i is clear that substantial or complete inhibition of carbohydrate breakdown gives n vegetalization comparable with what we know in the case of lithium.

In the case of *Echinarachnius*, controls in 0·1 % lithium chloride were run Practically all the embryos formed perfect exo-gastrulae.

As regards the phloridzin experiments little can be said. It is very insolubl in sea water, and though the concentrations named were attained by warming, i came out in crystalline form at the temperature necessary for normal developmen of the embryos. The actual concentrations must therefore have been lower tha those given in the table, and the fact that normal advanced gastrulae were obtaine loses much of its importance. This is particularly unfortunate since phloridzin i a specific inhibitor for phosphorylating glycolysis just as glyceraldehyde (in certai conditions) specifically inhibits non-phosphorylating glucolysis.

Summing up, it may be said, therefore, that at concentrations of *dl*-glyceralde

Table II. *Arbacia punctulata*, *T* 23° C.

Exp. 10:

	8 hr.	18 hr.	43 hr.	
Control	Vigorous blastulae	Prismatic gastrulae	Normal plutei	
Glyceraldehyde:				
10^{-2}	As control	—	—	no exo-gastrulae
10^{-3}	As control	As control	Retarded plutei with stumpy bulbous oral arms	
5×10^{-4}	As control	As control	As control	
10^{-4}	As control	As control	As control	
10^{-5}	As control	As control	As control	
Iodoacetate:				
3×10^{-2}	Morulae	—	—	no exo-gastrulae
10^{-2}	Late morulae	—	—	
10^{-3}	As control	As control	No further development	
5×10^{-4}	As control	As control	As control	
10^{-4}	As control	As control	As control	

Exp. 11:

	12 hr.	35 hr.	53 hr.	106 hr.	
Control	Early gastrulae	Normal plutei	Advanced plutei	Advanced plutei	
Glyceraldehyde:					
5×10^{-3}*	As control	No further development	—	—	no exo-gastrulae
10^{-3}*	As control	Stunted plutei	Stunted plutei and vigorous late gastrulae with tripartite gut and no spicules	The same	
5×10^{-4}	As control	As control	As control	—	
10^{-4}	As control	As control	As control	—	
Iodoacetate:					
3×10^{-2}	Morulae	Bun-shaped blastulae beginning to gastrulate	No further development	—	no exo-gastrulae
10^{-2}	Morulae	Mid-gastrulae	—	—	
5×10^{-3}	Morulae	No further development	—	—	
10^{-3}	As control	Abnormally shaped late gastrulae, no spicules	—	—	
5×10^{-4}	As control	Abnormally shaped late gastrulae, spicules appearing	—	—	

* 6 hr. treatment before fertilization only.

yde well within the range of maximal inhibition of carbohydrate breakdown in the issues of vertebrates, echinoderm embryos give no sign of exo-gastrulation or arked vegetalization. The same is true of iodoacetate. Controls show that the ame echinoderm material at the same time is fully capable of the marked vegetaliza- ion brought about by lithium. Hence the hypothesis of Lindahl, that the animal ole region is a centre of carbohydrate breakdown, and that vegetalization will ollow if this is inhibited, acquires no further plausibility from the present results. f the reduction in length of the oral arms seen in the highest concentrations of the hibitors is regarded as evidence of vegetalization, it must at any rate be admitted be very feeble as compared with what will be performed by lithium. Again, it is ossible that the mechanism of carbohydrate breakdown in the echinoderm embryo quite different from those which occur elsewhere and are known to be sensitive these inhibitors, but this is perhaps hardly very likely in view of the close similarity between the mechanisms in yeast, bacteria, and mammalian tissues. An whether the mechanism in the sea-urchin embryo is phosphorylating or non-phosphorylating does not matter, for iodoacetate would inhibit both and glyceraldehyde the latter only.

SUMMARY

Echinoderm embryos, cultivated in *dl*-glyceraldehyde and iodoacetate, a concentrations within the range of maximal inhibition of carbohydrate breakdow as established on other systems, show no sign of exo-gastrulation or marke vegetalization. How far this affects the hypothesis of the Scandinavian school tha the animal pole region in the echinoderm embryo is a centre of carbohydrate breakdown, is discussed in the text.

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STUDIES ON ADAPTATION TO SALINITY IN *GAMMARUS* SPP.

I. REGULATION OF BLOOD AND TISSUES AND THE PROBLEM OF ADAPTATION TO FRESH WATER

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(With Two Text-figures)

INTRODUCTION

NVASION of brackish and fresh waters by marine animals has not always been ccomplished by means of the same type of osmoregulatory mechanism. This is learly shown by the great differences in the ability of fresh-water animals to roduce a urine hypotonic to the blood (Table II) and to concentrate ions from the nedium (Krogh, 1939, p. 203). As a result the blood concentration is maintained t very different levels in different species (Table II). It should therefore be of nterest to investigate several species of the same genus which have become adapted o habitats of different salinity range and which, we can be reasonably certain, form n evolutionary series. For this reason we have chosen four species of the genus *ammarus*. In the present work a survey is made of the relation between the xternal medium and blood, when the salinity of the former is rapidly changed. n addition, estimations of tissue chloride show that the chloride in the tissues can e regulated against changes in that of the blood and that this mechanism is as nportant as that for regulation of blood concentration in facilitating adaptation to ianges of salinity.

In a later paper results of experiments will be recorded in which certain species are cclimatized by very gradual change to salinities not found in their normal habitats nd outside the range tolerated when transfer is rapid. Another interesting problem raised by the brackish water species *G. duebeni* which is apparently in the process f becoming adapted to fresh water and is actually the common inland water *ammarus* of Ireland (Reid, 1939). Comparative experimental work on samples of *. duebeni* from different habitats will be published in the second paper of this series. he *G. duebeni* used in the present experiments were taken from brackish water nly. The value of *Gammarus* as material for this kind of work is obviously limited y their small size, but ultramicro-methods are now available by which some useful formation can be obtained.

MATERIALS AND METHODS

G. locusta was supplied from Millport by Mr R. Elmhirst and was obtained by dredging. *G. obtusatus*[1] was collected under stones close to low-tide level a Cullercoats. Both these species were kept for at least 48 hr. in aerated sea water (540 mM.) before the experiments.

G. duebeni was collected from Meggies Burn, Blyth, Northumberland. The salinity of this stream is influenced by the daily tidal inflow but never becomes very low owing to saline water continuously pumped into it at its head from a coa mine nearby. Measurements made at irregular intervals during the past four years indicate that it fluctuates between about 10 and 50% sea water. They were kep at least 48 hr. in 50% sea water (270 mM.).

G. pulex collected from the Cor Burn, Northumberland, and kept in tap water

The above stock cultures were fed with elm leaves, but feeding was stopped during the experiments. Dilutions of sea water were made with tap water and aerated by thorough shaking. No further aeration was provided during the 24 hr taken for each experiment. To obtain the points in Fig. 1, about 25 animals were transferred direct from the culture to about 150 c.c. of dilute sea water of the required salinity, and the measurements were made after 24 hr. Except in the case of *G. pulex* (see below) only salinities were chosen in which the animals showed no signs of distress after this time.

Extraction of blood, after drying between filter papers, was effected by holding the animal between the thumb and forefinger of the left hand and pricking the dorsal vessel with a needle held in the right hand. A drop of blood immediately appears which can be sucked up into a waxed micropipette for transfer to the thermocouple loop for vapour-pressure determination, or directly into the graduated micropipette for chloride estimation.

Blood osmotic pressure was measured, to the nearest 2 mM./l., by Baldes's modification of the Hill vapour-pressure method (Baldes, 1934).

Blood chloride was estimated to the nearest 3 mM./l. by Wigglesworth's micromethod (Wigglesworth, 1938*a*). By the method adopted by Wigglesworth we found it impossible to obtain a stable coating of wax on the micropipettes and burettes. For the benefit of others who may find the same difficulty we suggest the following procedure by which we were invariably successful. With the object of removing possible moisture and adsorbed air from the inner surface, a column of absolute alcohol followed by one of xylol was drawn up the capillary. Immediately after discharging the latter, a column of molten wax was sucked up and discharged before the final coating operation. This was done as described by Wigglesworth except that the molten wax was as *cold* as possible.

Tissue chloride. The method used was based on Patterson's cold nitric acid digestion method for blood (Patterson, 1928). The main advantage of this method is its extreme rapidity. Five to ten *Gammarus* were dried on filter paper, cut in two nd the blood drained off by pressing between two filter papers. After weighing 100–200 mg.) they were transferred to a hard glass test-tube ($\frac{3}{4} \times 6$ in.) containing drop of distilled water, and were ground to a paste by means of a bulb-ended

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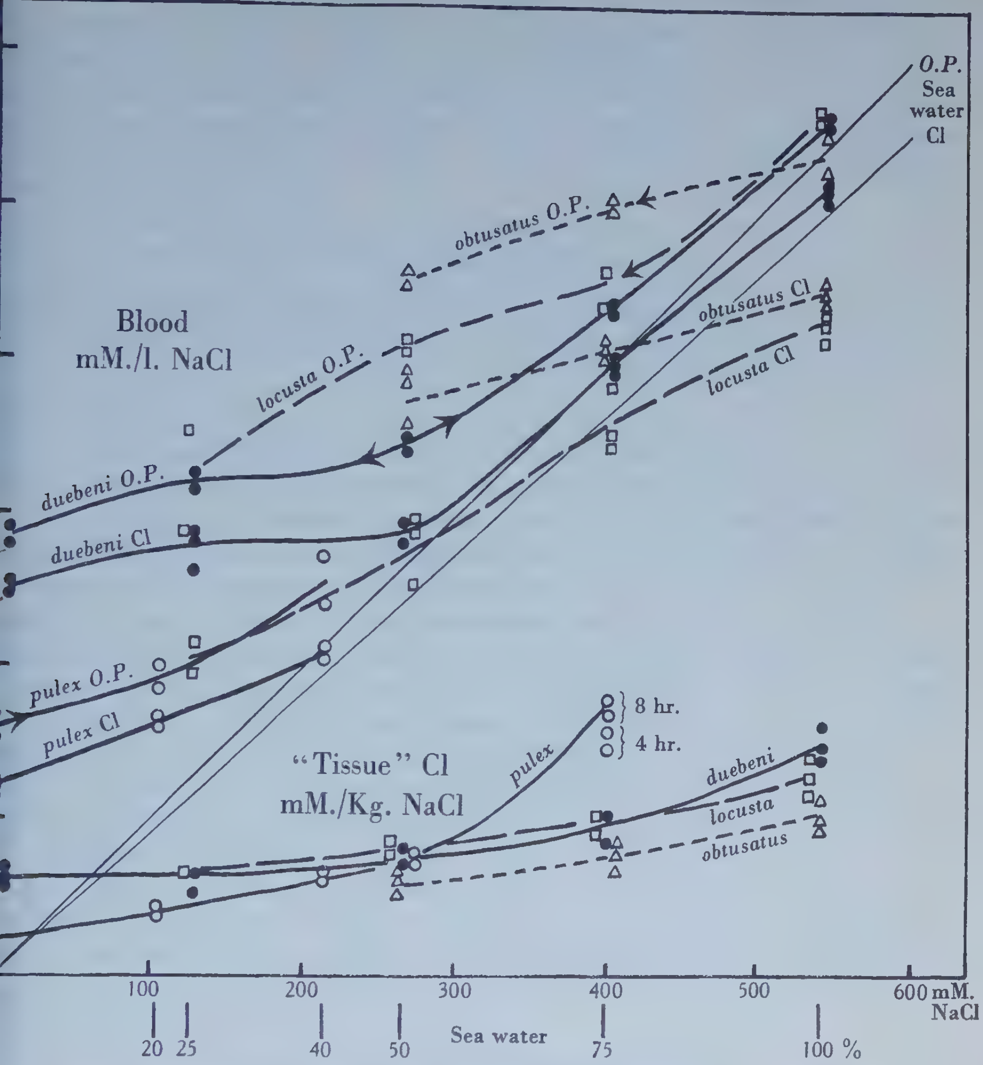
[1] A species allied to *G. marinus*, kindly identified by Mr G. M. Spooner of Plymouth.



ig. 1. Blood osmotic pressure (*O.P.*), blood chloride (Cl) expressed as mM. NaCl/l., and tissue loride expressed as mM. NaCl/kg. wet weight, 24 hr. after direct transfer to various concentrations sea water. The direction of transfer is indicated by the arrows on each osmotic pressure curve. With e exception of the highest concentration to which *G. pulex* was submitted (400 mM., see text), the nge of external concentrations chosen for each species was that which could be withstood without pparent harm for at least 24 hr.

lass rod. 3 c.c. of conc. HNO_3 were added and stirred thoroughly with the rod. fter a few minutes a clear yellow solution containing skeletal debris was obtained. he rod was washed into the tube with the least possible distilled water from a wash bottle. 1 or 2 c.c. (according to the amount of Cl present) of 0·25 % $AgNO_3$ were added and the mixture shaken, followed by 3 c.c. of pure acetone. After thoroughly cooling under the tap the excess $AgNO_3$ was titrated with 0·05 *N* NaSCN from a Bang 2 c.c. microburette, using 6 drops of saturated iron alum as indicator. The NaSCN was previously standardized by substituting for the tissue 1 c.c. of *c.* 8·4 mM NaCl, this being the only originally accurate standard required. The method was tested on a piece of fresh fish muscle (haddock), and the following seven results were obtained: 33, 31, 34, 34, 33, 33, 31 mM./kg.Cl. These figures give a standard deviation of ± 1·6 %.

DISCUSSION OF RESULTS

The main results are shown in Fig. 1. These curves require no detailed description since they are largely self-explanatory. We will therefore proceed to discuss their more significant features.

(i) *The marine species,* G. obtusatus *and* locusta

Both of these can withstand considerable dilution of the medium, and over their entire tolerance range maintain their blood concentration at a higher level than *G. duebeni*, which can actually survive much greater dilution. In fact, the degree of dilution which each of these three species can tolerate bears an inverse relation to the osmotic pressure of the blood. In view of the wider tolerance range of *G. locusta* than that of *G. obtusatus* it is interesting to note that *locusta* has often been recorded from brackish water, whereas *obtusatus* has never been found except on shores inundated by pure sea water (private communication from Mr G. M. Spooner).[1] It is well known that *G. marinus* commonly occurs in brackish water at the mouths of streams and estuaries. Though its tolerance range has not, so far as we know been determined, Widmann (1935) was able to acclimatize it to 186 mM. sea water in which its blood concentration was 470 mM. Its osmoregulatory mechanism is thus even more effective than that of *G. locusta* (Fig. 1). But since some marine animals such as *Arenicola marina* can survive in 25 % sea water with no mechanism for maintaining hypertonic body fluids (Krogh, 1939, p. 47,) the possession of such by the marine *Gammarus* species does not appear to be functionally significant.

(ii) *The brackish water species,* G. duebeni

This species can be transferred direct from 100 to 2 % sea water and vice versa without harm. It will be shown in a subsequent paper that adaptation to extreme dilutions and to fresh water depends upon the composition of the water. From the form of the blood curve (Fig. 1) it is evident that there is a critical external concentration (259–350 mM.) below which the osmoregulatory mechanism comes into play, and above which the hypertonicity of the blood is relatively slight.

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[1] Howes (1939) found an abnormal form of *G. locusta* in a brackish lagoon in Essex, of which the salinity fluctuated between 21·5 and 27·9 ‰ (*c.* 370–480 mM.).

(iii) *The fresh-water species*, G. pulex

The blood concentration is here much lower than in the other species. Widmann 1935) measured the freezing-point of the blood of *G. pulex* in fresh water, by ccumulating blood samples from a large number of animals. The figures do not ntirely agree with ours. She found seasonal differences in blood concentration anging from 185 mM. in August to 265 mM. in February. Our figures, obtained n April, were lower than these (142–165 mM.). The reasons for this seasonal fluctua-on, which we have not yet confirmed, are quite obscure, but we are not at present oncerned with them since all our experiments were done at the same time of year n animals with a similar level of blood concentration.

G. pulex can withstand sudden transfer to 20% sea water (107 mM.), but in 15 mM. a few died after 24 hr., though the majority remained normal. This pecies showed more individual variation than the others. In 50% sea water 270 mM.) all had practically stopped moving after 24 hr. The blood concentration s raised by increasing the salinity of the medium, and a critical point seems to be eached when the concentration of the medium approaches that of the blood Fig. 1). Widmann (1935) acclimatized *G. pulex* by gradual addition of sea water uring 17 days to 155 mM. sea water. At this season (May) the blood concentration n fresh water was *c.* 220 mM. and at the end of the experiment it had risen to 83 mM. These figures would fall on a curve parallel with ours but at a higher level.

(iv) *Blood chloride*

The curves for blood chloride (Fig. 1) are mainly parallel with those for osmotic ressure. This suggests that the change in total concentration is due to salt exchange nd not to movement of water under osmotic forces. The absence of any significant hange in total water content was also shown by weight measurements on *G. pulex* nd *duebeni* following a sudden change in external salinity. These are given in able 1. The difference between total osmotic pressure and chloride concentration non-Cl fraction) is relatively constant for each species, but there is an obvious ifference between the bloods of the two marine species and those of *G. duebeni* nd *pulex*. The approximate average concentrations of the non-Cl fractions are as llows: *G. locusta* 120, *obtusatus* 95, *duebeni* 30, and *pulex* 35 mM. It remains to be een whether a low non-Cl fraction in *G. duebeni* and *pulex* is in any way connected ith their ability to live in low salinities.

Table I

G. duebeni Previously kept 24 hr. in 540 mM. sea water. Transferred to 10 mM. 10 specimens, original wt. 376 mg.		*G. pulex* Previously kept in tap water. Transferred to 215 mM. sea water. 20 specimens, original wt. 638 mg.	
Time hr.	Wt. % original	Time hr.	Wt. % original
0·6	100	9·5	99
1·2	100	22·5	98
9·2	98	48·0	98
22·5	97	—	—

(v) *Tissue chloride*

The blood curves in Fig. 1 present a problem which cannot be entirely solve by reference to blood-regulating mechanisms. Why should the two marine specie begin to succumb in dilute media before the blood concentration has fallen to level normally maintained by *G. pulex*? And, conversely, why should *G. pulex* b unable to withstand a blood concentration as high as the lowest level maintaine successfully by the brackish and marine species? Regulation of the blood is o course important only in so far as it leads to the maintenance of healthy tissues Tissues in general, though in osmotic equilibrium with the blood, are known t differ considerably from it in the concentration of individual ions, a condition whic is essential for the normal functioning of the cells. It may therefore be suspecte that the intracellular mechanism for maintaining these differences depends upon certain range of blood concentration which differs in each species. For this reaso we estimated tissue chloride, which in most tissues is at a much lower concentratio than in the blood. In order to detect a breakdown in the intracellular regulator mechanism it is necessary to measure the tissue Cl of animals in salinities whic they are unable to withstand, but at the same time the concentration of the bloo must not be allowed to depart from the range which is normal for other species There are practical difficulties in this, since from unhealthy animals succumbin to the effects of low salinity treatment, it was impossible to obtain a discrete dro of blood which could be picked up in a micropipette. No doubt this is due to a los of hydrostatic pressure. Though the blood could not be extracted in the manne necessary for vapour pressure or chloride estimations, it was readily drained off a a preliminary to tissue chloride estimation (see section on methods). For thi reason we could not determine the blood concentration of the matine species i water of salinity lower than the tolerance range. But with *G. pulex*, in salinitie above the normal tolerance range, the blood concentration could be predicted wit certainty as being at least within the range withstood by *G. duebeni* (Fig. 1).

The tissue Cl concentration found for *G. pulex* in fresh water is of an order t be expected from previous work on the muscles of fresh-water animals (Table II) The differences are obviously related to the differences in blood chloride. Scholle (1933) found 152 mM./kg. Cl in the muscle of *Eriocheir sinensis* in sea water, whic is almost identical with our average figure for *G. duebeni* in sea water. Though th blood and tissue Cl concentrations are not exactly comparable since they wer estimated per litre and per kg. respectively, yet, with the greatest possible allowanc for the lower water content of the tissues, it is evident that a very large blood tissu Cl gradient is maintained within the tolerance range of all species. *G. pulex* wa presumably evolved from a brackish water ancestor, but it is now less able tha *G. duebeni* to maintain a low tissue Cl when the blood Cl is rapidly raised. O transfer to 40% sea water (215 mM.) the blood Cl is increased by about 56% an the tissue Cl by about 140%. This is not sufficient to immobilize the animals (onl healthy ones were analysed), but in 50% sea water (270 mM.) the still more rapi increase of tissue Cl is associated with obviously deleterious effects on all th

Table II. *Fresh-water animals*

Blood and urine figures converted where necessary to mM./l., tissue Cl quoted as mM./kg. wet weight

	Blood		Urine	Tissue	Authority
	O.P.	Cl	O.P.	Cl	
Crustacea:					
Eriocheir sinensis	318	280	330	43 (muscle)	Scholles (1933)
Telphusa fluviatile	340	—	342	—	Schlieper & Herrmann (1930)
Potamobius fluviatilis	238	189	47	37 (muscle)	Scholles (1933)
Cambarus clarkii	188	117	19	—	Lienemann (1938)
Gammarus pulex	160	120	—	25	This paper
	185–263	—	—	—	Widmann (1935)
Asellus aquaticus	265–302	—	—	—	Widmann (1935)
Daphnia magna	68	—	—	—	Fritsche (1916)
Insecta:					
Sigara distincta	183	—	—	—	Claus (1937)
Aedes aegypti (larva)	138	40	—	—	Wigglesworth (1938*b*)
Aedes detritus (larva)	140	35	—	—	Beadle (1939)
Mollusca:					
Anodonta cygnea	16	—	10	—	Picken (1937)
Limnea peregra	73	—*	54	—	Picken (1937)
Fish:					
Amia calva	158	—	21	—	Smith (1932)
Cyprinus carpio	147	—	—	—	Duval (1925)
Amphibia:					
Frog	115	76	—	—	Macallum (1926)
	129	—	50†	—	Botazzi (1908)
	—	—	—	19 (muscle)	Hill & Kupalow (1930)

* Wigglesworth's observations make it practically certain that the urine is hypotonic to the ood.

† Overton (1904; quoted by Krogh, 1939, p. 155) found that at 0° C. the osmotic pressure of the rine is less than one-tenth that of the blood.

nimals. So rapid is the penetration of chloride into the tissues in 75 % sea water 400 mM.) that in 4 hr. the tissue Cl had already reached the level finally reached y *G. duebeni* in 540 mM. sea water. After 8 hr. a further rise had occurred, and the nimals had almost ceased moving. It has been maintained that in frog muscle the hloride is confined to the extracellular fluid which is in equilibrium with the blood Eggleton *et al.* 1937). Whether or not this is true of *Gammarus* tissues is unimportant in the present connexion. The conception of a blood/tissue Cl gradient is qually valid, and a higher percentage increase in the tissues as a whole than in the lood can only be explained as due to intracellular penetration of chloride.

On the assumption that the tissues remain isotonic with the blood, it is possible calculate from the data in Fig. 1 the percentage of the total tissue osmotic ressure due to Cl. The results are plotted in Fig. 2 against blood Cl. No previous orrection was made to the tissue Cl figures (estimated per kg.) for the water ntent of the tissues, which may vary, but presumably to the same extent in each species over the same range of blood concentration. These curves illustrate clearly the importance for adaptation to changing salinity of the mechanism for regulating tissue composition against changes in the blood. The two species *G. duebeni* an *locusta*, which have the widest tolerance range, have the greatest powers of regulation, whilst the tissues of *G. pulex* can be maintained only with a relatively low blood chloride.[1] Whether the restricted range of *G. obtusatus* is due to inability t adapt its tissues to a low blood concentration, or to insufficient power to maintain hypertonic blood in dilute sea water cannot be decided from the data available

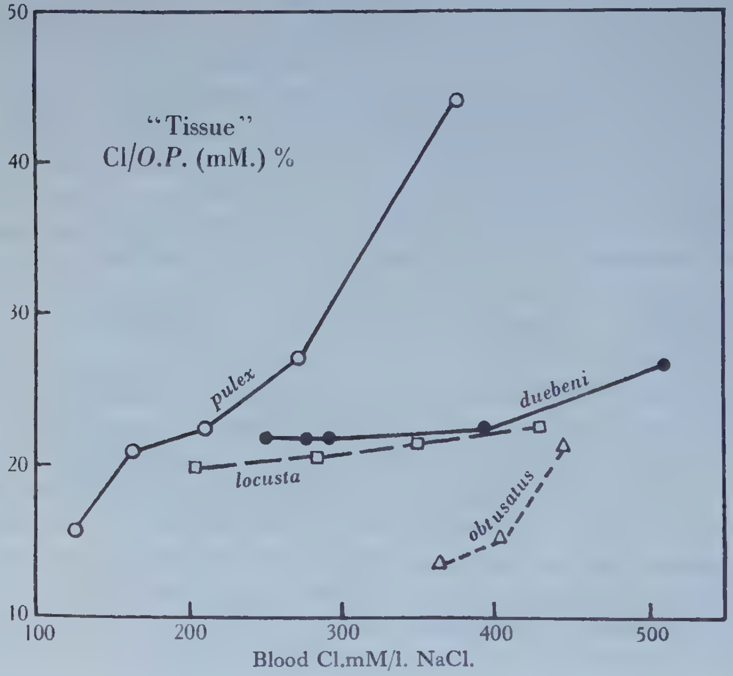

Fig. 2. The relation between blood chloride (mM. NaCl/l.) and the ratio tissue Cl/tissue osmoti pressure (%), calculated from the data in Fig. 1. It was assumed that the tissues remain isotoni with the blood, and no correction was made for the water content of the tissues, whose Cl wa estimated as mM. NaCl/kg. wet weight.

Owing to the roughness of the method for obtaining tissues for Cl estimation further speculation on the meaning of the curves in Fig. 2 would be useless. I would, however, be worth while doing similar work on large animals such as crabs from which a sufficient quantity of a single tissue could be got for estimations o several ions.

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[1] The highest percentage Cl reached by *G. pulex* may actually be greater than that calculate (44 %), since the animals had been in 400 mM. sea water for only 8 hr. It is possible that the tissue had not yet become isotonic with the blood or that the latter had not yet come into equilibrium wit the external medium, both of which were assumed in making the calculation.

ADAPTATION TO FRESH WATER

It is remarkable that fresh-water animals differ so greatly in the concentration
f their bloods. We have collected as much data as possible to illustrate this
Table II). These variations may be due in part to mere quantitative differences
etween the osmoregulatory mechanisms. But that these mechanisms differ also
n kind is clearly shown by the available data for urine concentrations (Table II).
he urine is hypotonic to the blood in all except the crabs, *Eriocheir sinensis* and
elopusa fluviatile, in which it is iso- or slightly hypertonic to the blood. It is
urprising that these two species, in which the excretory organs do not therefore
unction for osmoregulation, happen to be those with the highest blood concentra-
ion (over 300 mM.). It is known that *Carcinus moenas* also produces an isotonic
rine (Schlieper & Herrmann, 1930). In dilute sea water it maintains a strongly
ypertonic blood (of the same order as that of *G. locusta*), and, though apparently
ncapable of adaptation to pure fresh water, interpolation of the blood concentration
urve would give a concentration in fresh water of about 300 mM. (Duval, 1925).
f a high blood concentration in dilute sea water is associated with iso- and a low
lood concentration with hypotonic urine production, we should expect from our
esults (Fig. 1) that the marine *Gammarus* species are of the former and *G. pulex*
f the latter class. There is, of course, no direct evidence on this point, but Schwabe
1933), from a comparison of the structure of the excretory organs of *G. locusta*
nd *pulex*, deduced that only *G. pulex* can produce an hypotonic urine. A similar
nvestigation of *G. duebeni* would be of interest.

We suggest the following as a working hypothesis to explain the facts: adaptation
o fresh water has proceeded by two main stages. The first was accomplished by a
nechanism which is very effective in maintaining a high blood concentration. The
xcretory organs do not play a part in this mechanism, which probably entails
ctive absorption of ions from the medium. By this means the tissues are not
ubjected to a very great change in blood concentration. The second stage was
narked by a lowering of the blood concentration with a consequent lessening of
oth blood/external medium (O.P.) and blood/tissue Cl gradients to levels more
asily maintained. This was accompanied by the development of the renal salt
eabsorption mechanism, which further facilitated adaptation to fresh water. The
ttainment of this final condition entails the inability to withstand transfer back to
nore than low concentrations of sea water, for the reason that the tissues have lost
he power of maintaining a high blood/tissue Cl gradient, which is essential for
esistance to a raised blood concentration (e.g. *G. pulex*, Fig. 1). Duval (1925)
oted that not only *Eriocheir sinensis*, which is also found in brackish water, but also
elphusa fluviatile, a purely fresh-water form, can be transferred from fresh water
irect to sea water without harm, the blood of both becoming ultimately isotonic
vith the medium. Both of these maintain high blood concentrations and, as shown
y *Eriocheir* (Table II), high blood/tissue Cl gradients. On the other hand, most
resh-water animals have a low blood concentration and presumably, as in the cases

of *G. pulex* and the frog (Table II), a low blood/tissue Cl gradient. This, according to our argument, would account for their restricted salinity tolerance ranges *Potamobius fluviatilis* has an intermediate blood concentration level (Table II). I is therefore significant that Bogucki (1934) found it to withstand a salinity as high but not higher than *c.* 370 mM., in which its blood concentration was approaching that of the medium.

Duval (1925), in comparing *Telphusa fluviatile*, the carp, and *Anodonta cygnea* did in fact conclude that the degree of salinity which a fresh-water animal can withstand depends upon the level of its blood concentration. Whilst confirming this we would suggest further that high blood concentration is associated with an intracellular mechanism for maintaining a high blood/tissue Cl gradient, and that it is upon this that the tolerance range primarily depends. The inequality in the distribution of ions is certainly not confined to Cl, but tissue Cl was chosen for the ease with which it is estimated. The way is clear, by carefully controlled experiments for further testing of this hypothesis. If the seasonal differences in blood concentration of *G. pulex* found by Widmann (1935) can be confirmed, it would be of great interest to determine whether these are associated with differences in the salinity tolerance range. There are several recorded experiments in which, by very gradual change over several months, purely marine animals have been acclimatized to fresh water and vice versa, and Sexton (1928) succeeded by these means in adapting *G. pulex* to sea water and *G. locusta* to fresh water. The physiological mechanism by which this is accomplished is obviously of great interest, and we are starting investigations on this point.

There are of course several animals of fresh-water origin which can live in extremely saline water. But, as investigations on two of such animals show (*Artemia salina*, Medwedewa, 1927; *Aedes detritus* larva, Beadle, 1939), a special mechanism has been evolved whereby the blood concentration is maintained at a level characteristic of purely fresh-water animals. By these means they can withstand a salinity several times that of sea water, conditions which are intolerable to most marine animals. A similar mechanism also exists in a few brackish water animals, by which the blood concentration is kept at a high level (e.g. 330–350 mM. in *Palaemonetes varians*, Pannikar, 1939), though hypotonic to the medium when this is pure sea water. Whether such animals can withstand an abnormally high salinity has not apparently been determined.

SUMMARY

1. Four species of *Gammarus* were studied: the fresh-water *G. pulex*, the brackish water *G. duebeni*, and two normally marine species *G. locusta* and *obtusatus*, the former of which has also been recorded from brackish water.

2. The relation between osmotic pressure and chloride of the blood and of the external medium, after sudden transfer to salinities which could be withstood for at least 24 hr., is shown in Fig. 1.

3. The changes in blood osmotic pressure are due to salt and not to water movements.

4. The marine species *G. obtusatus* and *locusta* maintain a very hypertonic lood in dilute sea water and can withstand 50% (270 mM.) and 25% (135 mM.) ea water respectively.

5. The brackish water *G. duebeni* has a tolerance range from pure sea water to vater containing a trace of salt, but is not as well adapted to fresh water as *G. pulex*.

6. For a wide salinity tolerance range two mechanisms are necessary, (*a*) for egulating the blood concentration within certain limits, and (*b*) for maintaining a ow intracellular concentration of certain ions (e.g. Cl) in spite of changes in blood oncentration. Defection of the latter mechanism can alone account for the inbility of *G. pulex* to withstand direct transfer to more than about 40% sea water 115 mM.).

7. On the basis of this work and that of others on other animals the following ypothesis is suggested. Adaptation to fresh water has proceeded by two main tages: (*a*) Probably by active ion absorption, a high blood concentration is mainained (as in *Eriocheir sinensis* and *Telphusa fluviatile*) and is associated with a large lood/tissue Cl gradient. Such animals can still be transferred suddenly to a high oncentration of sea water. (*b*) Evolution of the renal salt-reabsorption mechanism, nd lowering of both blood concentration and blood/tissue Cl gradient to levels nore easily maintained (as in *G. pulex* and most fresh-water animals). The consequent loss of power to maintain a large blood/tissue Cl gradient entails inability o withstand transfer to more than low concentrations of sea water, unless, as in ertain species, a special mechanism is evolved for preventing the blood concentraion from rising.

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THE EFFECT OF TESTOSTERONE PROPIONATE ON THE SEX-LIFE OF THE FEMALE RAT

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The action of hormonic substances on the smaller experimental animals is usually assessed by killing the animal and examining its organs shortly after treatment. The present paper deals with a study of the effects of testosterone propionate injections on the subsequent life of the female rat.

An earlier paper (Groome, 1939, which see for references) describes the immediate anatomical effects of testosterone propionate on the young female rat. In this instance the experiment was repeated, but instead of killing the females, when the course of injections was completed, males were introduced and the sexual cycle observed.

MATERIALS AND METHODS

Eight female rats 55 days of age and four of 74 days were divided into two groups. One group was kept for control purposes, while the six animals in the other group received subcutaneous injections of 500γ testosterone propionate daily for 8 days. On the 9th day, when the animals were 64 and 83 days of age, adult males were introduced into the cages. Vaginal smears were taken until pregnancy, or until the cycle of pseudopregnancies, had been established. The young of the experimental animals were killed and examined; but the young of the controls were kept to replenish breeding stock, and as a result two small litters were unfortunately eaten by their parent before their sex had been noted. The twelve females with which the experiment started were all killed 60 days after the original introduction of the males, which will subsequently be called the 1st day of the experiment.

RESULTS

Oestrus was observed in the controls from the 1st to 4th day, and in each case fertile matings were made. The young were born between the 23rd and 26th day, the known sex ratio being 100 : 100. Some of the young and all the controls were killed on the 60th day and were found to be normal. The controls, weighed to the nearest 5 g., had an average body weight of 179 g.

Oestrus was much delayed in the experimental animals. The first oestrus of nos. 3, 4 and 6 was not observed from vaginal smears, but deduced from the presence of sperms in the vagina. In nos. 1 and 5 oestrus was delayed by 16 and

2 days respectively, when fertile matings were obtained. No. 2 became oestrous on he 8th day, when a sterile mating delayed the next oestrus till the 22nd day on which a fertile mating occurred. No. 3 experienced two pseudopregnancies between he 7th and 34th day when she conceived. Nos. 4 and 6 had a sequence of pseudo-pregnancies, from the 9th and 7th day respectively, until they were killed on the oth day. The vaginal smears of all these animals at first contained mucus only, hen mucus and leucocytes and eventually, as the mucus disappeared, the typical ro-oestrous picture was obtained. A number of attempted copulations were noted when the vagina was in the mucous condition.

Table I. *Experimental animals*

	Days of age when male introduced	Oestrus observed days	Young born days	Young males	Young females	Wt. at death 60 days (g.)
1	64	16	39	1	0	155
2	64	8.22	44	7	4	170
3	64	7.(20).34	56	4	3	185
4	64	9.23 *et seq.*	—	0	0	185
5	83	12	34	2	3	195
6	83	7.21 *et seq.*	—	0	0	210
			Controls			
1	64	3	25	3	3	170
2	64	3	25	3	3	170
3	64	2	24	3	2	185
4	64	3	25	3?		170
5	83	4	26	2?		195
6	83	1	23	2	2	185
2nd litter	—	—	57	3	4	

Columns 2, 3 and 6 refer to the number of days after the introduction of males.

The young were born between the 34th and 56th day, the sex ratio being 40 : 100; it will be seen from Table I that this ratio depends upon the abnormal itters of nos. 1 and 2. All the young were dissected and found to be normal, there being no tendency to intersexuality. The experimental females, killed on the 60th lay and weighed to the nearest 5 g., had an average body weight of 183 g.

All the experimental females had hypertrophied and abnormally vascular litoris-like organs, which were made prominent by the enlarged female preputial lands. The enlargement of the mammary apparatus was consistent with previous regnancies or pseudopregnancies. The vaginae were normal to the cyclic condition f each individual. The uteri were indistinguishable from those of the controls, vith the exception of nos. 4 and 6. The uteri of nos. 4 and 6 had the plicated ppearance, typical of testosterone propionate activation, with reduced epithelial nd glandular cells, but showing considerable thickening of the muscle and mucosa. The ovaries of all these animals were apparently normal and contained Graafian ollicles and corpora lutea.

DISCUSSION

The course of testosterone propionate injections given to young female rats was the same as that described in an earlier paper; the immediate effects of the injections (Groome, 1939) can therefore be compared with their action over a period of 60 days.

The injections cause an immediate increase in body weight, but this is not maintained after 2 months.

The ovaries are activated by testosterone propionate, but after pseudopregnancies or pregnancy they are indistinguishable from normal.

In the earlier paper hypertrophy of the uterus with a progestational appearance, which might be genuine pseudopregnancy, is reported. In this experiment the cycle of pseudopregnancies was retained in the presence of the male until terminated by pregnancy. It will be noted that in the two animals which failed to become pregnant, the typical progestational type of uterus was retained for 60 days.

The injections caused vaginal mucification, which disappeared within 6 days in all cases.

The mammary glands are apparently unaffected, but the preputial glands and clitoris-like organ remain enlarged for 60 days. It appears therefore that the ovary will maintain the hypertrophied preputial gland; and that the clitoris-like organ will maintain its increased length long after the injection of androgens has ceased.

Testosterone propionate will delay oestrus by from 6 to 12 days, and pregnancy is delayed by from between 11 and 30 days to 60+ days. The sex ratio of the young born to treated females was abnormally high, but as this figure depends on one large litter only it has no significance.

It is of course impossible to be certain that each individual receives the same dose by injection, but it seems probable that there is considerable variation in individual sensitivity to testosterone propionate. It may be recalled that in the earlier experiment the injections caused "hypertrophy of the uterus, in one case having an oestrous, and in the others a progestational appearance". The individual differences here recorded are however differences of intensity rather than of fact.

From the point of view of the time factor the clitoris-like organ and preputial glands are most affected by testosterone propionate; the uterus is intermediate, and the vagina is least affected by it. The effect on the ovaries is more difficult to determine. It was suggested in the earlier work that they were activated by testosterone propionate, but that their effect on the principal accessory organs was masked by the direct action of the injection. The normal cyclic changes of the ovary are evidently not disorganized for long, as shown by the recurrent oestrus and pseudopregnancy. The failure to conceive was probably due to the condition of the uterus alone. On the other hand, the apparent willingness of both sexes to attempt copulation, when the vagina was anoestrous and contained mucus, may indicate that the normal ovarian cycle was not impaired; for there is no reason to suppose that the mere cornification of the vagina provides the stimulus to copulate or either sex. It can, however, be stated that the injections temporarily remove the control of cyclical changes in the uterus and vagina from the ovary, which may itself retain an undisturbed cycle.

SUMMARY

Males were introduced into the cages of six young female rats, which had been njected with 500γ testosterone propionate daily for 8 days.

Oestrus was delayed by from 6 to 12 days and was followed by recurrent pseudopregnancies or pregnancy. The young were sexually normal and sex-ratio was not significantly affected.

The injections caused vaginal mucification, which disappeared within 6 days.

These animals (and six controls) were killed on the 60th day when it was found that:

(1) There was no abnormal increase in body weight or in the mammary glands. The ovaries appeared normal.

(2) There was hypertrophy of the preputial glands and clitoris-like organ.

(3) The uteri of two animals that had been recurrently pseudopregnant, but not pregnant, had the typical plicated progestational appearance of testosterone propionate activation.

It is suggested that the delay in conception in all cases was due to the condition of the uterus, which underwent a cycle of pseudopregnancies (possibly initiated by the injections) in the presence of the male, until this cycle was terminated by pregnancy.

It is also suggested that the treatment temporarily removes the control of cyclical changes in the accessory organs from the ovary, which may itself retain an undisturbed cycle.

My thanks are due to Messrs Ciba, Ltd., who kindly supplied the whole of the testosterone propionate (Perandren).

REFERENCE

GROOME, J. R. (1939). *Quart. J. exp. Physiol.* **29**, 367.

THE EFFECTS OF CASTRATION BY PARASITES AND X-RAYS ON THE SECONDARY SEX CHARACTERS OF PRAWNS (*LEANDER* SPP.)

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(With One Plate and Five Text-figures)

I. THE EFFECTS OF CASTRATION ON THE PERMANENT DIFFERENCES BETWEEN THE SEXES

Introduction

In 1837 Rathke made the observation that, in prawns of the genus *Palaemon* (*Leander*), only the females were parasitized by *Bopyrus*. Subsequently Giard, in 1888, pointed out that there is an alternative explanation for this remarkable observation: that any males which are parasitized show the phenomenon of sex reversal ("castration parasitaire") and take on the form of females. Giard himself examined some parasitized specimens of *Leander* (unfortunately he is not explicit as to which species) and found that his presumption had been correct. He notes however, as an exception, that one male of *L. serratus* showed no modification of the secondary sex characters. Giard also cites the observation of Hansen that in the genus *Hippolyte* only females are parasitized, and this he explains in the same way.

That the Epicarids can cause sex reversal of their male hosts is known from two other examples. Bonnier, in his monograph on the Bopyrids (1900), gives an account of the case of *Galathea intermedia* parasitized by *Pleurocrypta galatheae*, illustrating the modifications of the abdominal appendages of the male towards the female type. More recently we have the excellent paper of Tucker (1930), which describes the effects of *Gyge branchialis* on *Upogebia littoralis*, and this paper goes deeply into the general relations of the Epicarids with their hosts and discusses in all its aspects the problem of sex reversal due to the action of parasites.

During my tenure of the Oxford Biological Scholarship at the Zoological Station, Naples, 1938–9, I noticed that two species of *Leander* occurring there, *L. xiphias* Risso and *L. squilla* (L.), were very frequently parasitized by two species of *Bopyrus*, *B. xiphias* Giard & Bonnier and *B. Helleri* Giard & Bonnier respectively, and I thought that with such abundance of material it would be of value to make a detailed investigation of the conditions obtaining in these species. I would like here to express my great indebtedness to Dr R. Dohrn and the staff of the Zoological Station for their kindness and help at all times during my stay in Naples.

Observations

The general relations existing between *Leander* and its parasite *Bopyrus* resemble hose described by Tucker (1930) for *Upogebia* and its parasite *Gyge*. Thus, *Bopyrus* s a gill-chamber parasite sucking blood from the branchiostegite, taking up a osition on the right or left side and developing a bilateral asymmetry accordingly. 'he dwarf male is almost always to be found with his larger female partner, and it s only in the earliest stages of parasitic life that the female is to be found alone. ıbout 5 % of the host species are infested and, in upwards of 500 examined, four pecimens were carrying a *Bopyrus* in both gill chambers.

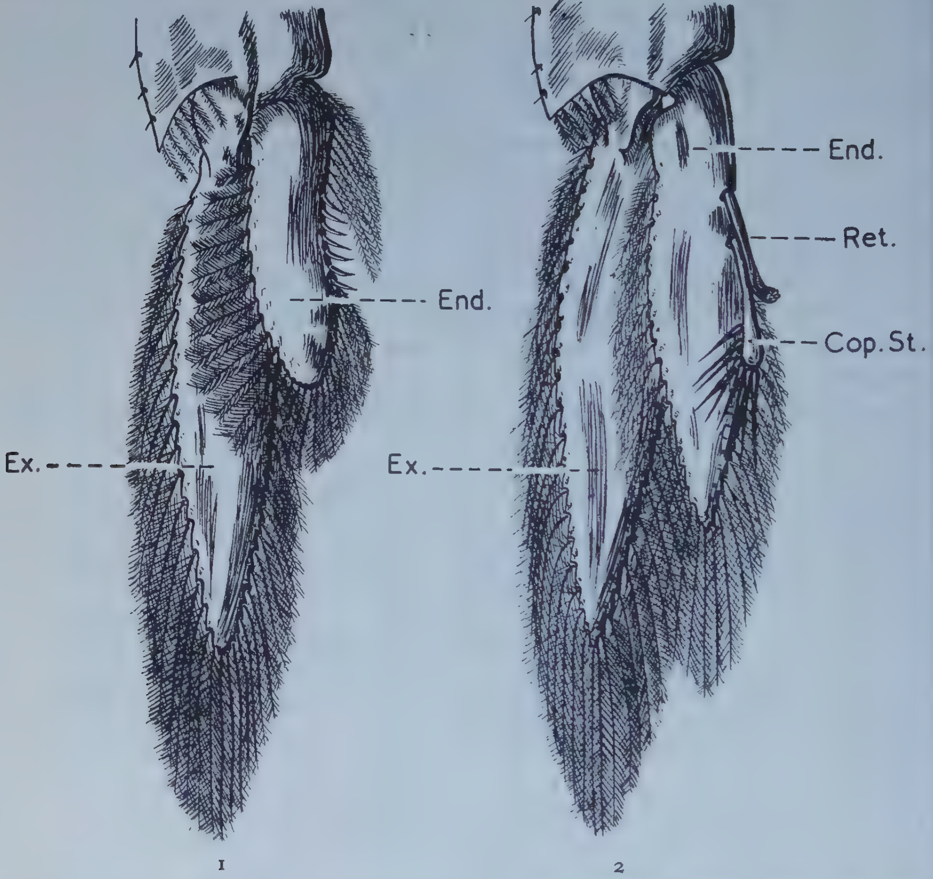
The presence of *Bopyrus* does not prevent *Leander* from moulting, in fact arasitized females at the breeding season moult much more frequently than do nparasitized individuals of the same sex at this time. The parasite has, furthermore, ttle or no effect on the final size attained by the host. Parasitism begins when the ost is very small (when the overall length from the tip of the rostrum to the tail is pproximately 15 mm.) and, as Tucker found in *Upogebia*, so close is the correlation etween host size and parasite size that one must assume that the initial attachment s confined to this stage.

Giard has summarized the features which distinguish the sexes externally in *eander*:

Male	Female
The endopodite of the first pleopod is relatively longer	Relatively shorter
For the middle third of its length the internal border of the endopodite of the first pleopod bears a row of small, often recurved and heavily chitinized hairs	Throughout its length, this endopodite bears long, unspecialized, feathered hairs
The endopodite of the second pleopod bears on its internal border a retinaculum and a copulatory style armed with stiff spines	This endopodite, as all the remainder, bears only a retinaculum
The internal branch of the external ramus of the first antenna, which bears the olfactory hairs, is relatively longer and bears these hairs in greater number.	Relatively shorter, and bearing fewer hairs
The chelae are relatively longer	Relatively shorter
The free space between the bases of the fifth pair of pereiopods is relatively narrower	Relatively wider
The maximum size reached is absolutely smaller	Absolutely greater

Giard notes that the characters which show definite modification in the female irection in parasitized male *Leander* are: endopodite of the first pleopod (reduced n size, and becoming of female shape without specialized hooks), copulatory style reduced in size), free space between the bases of the fifth pair of pereiopods (someimes widens). I have dealt mainly with the first two of these characters.

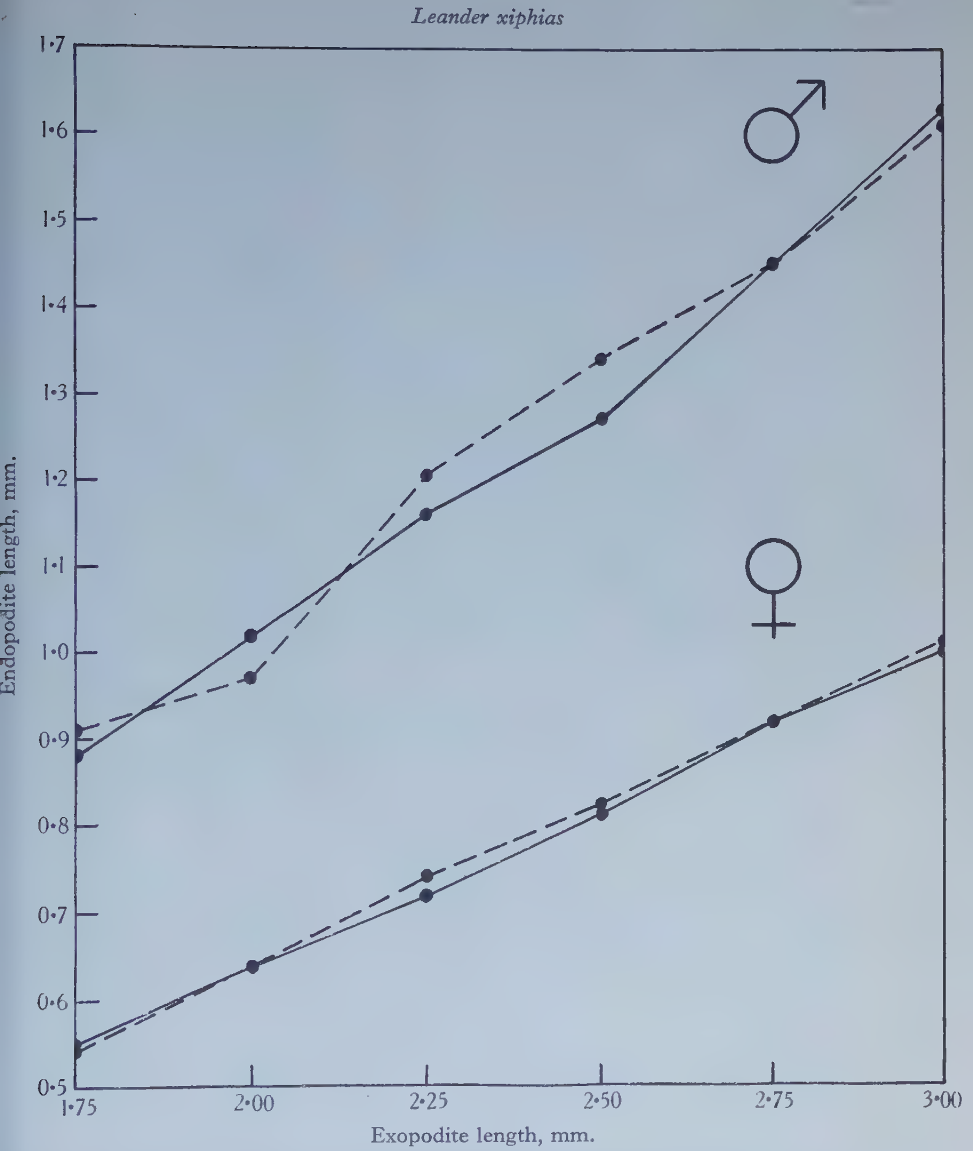
At first I made a point of determining the sex of a parasitized *Leander* from xamination of its gonad. However, I soon found that the sexes could be readily istinguished, whether parasitized or not, by the length and shape of the endpodite of the first pleopod (see Text-figs. 1 and 4). Out of about 300 parasitized pecimens scored as females by this character, there was not a single individual which showed signs of a copulatory style on the second pleopod (see Text-fig. 1, 2). Subsequently I made a series of relative length measurements for the endopodite and exopodite of the first pleopod in males, parasitized males, females and parasitized females of both species. The exopodite measurements were lumped into size groups of 0·25 mm. interval. Averages of endopodite length for each of the exopodite size groups were used in the construction of the two accompanying



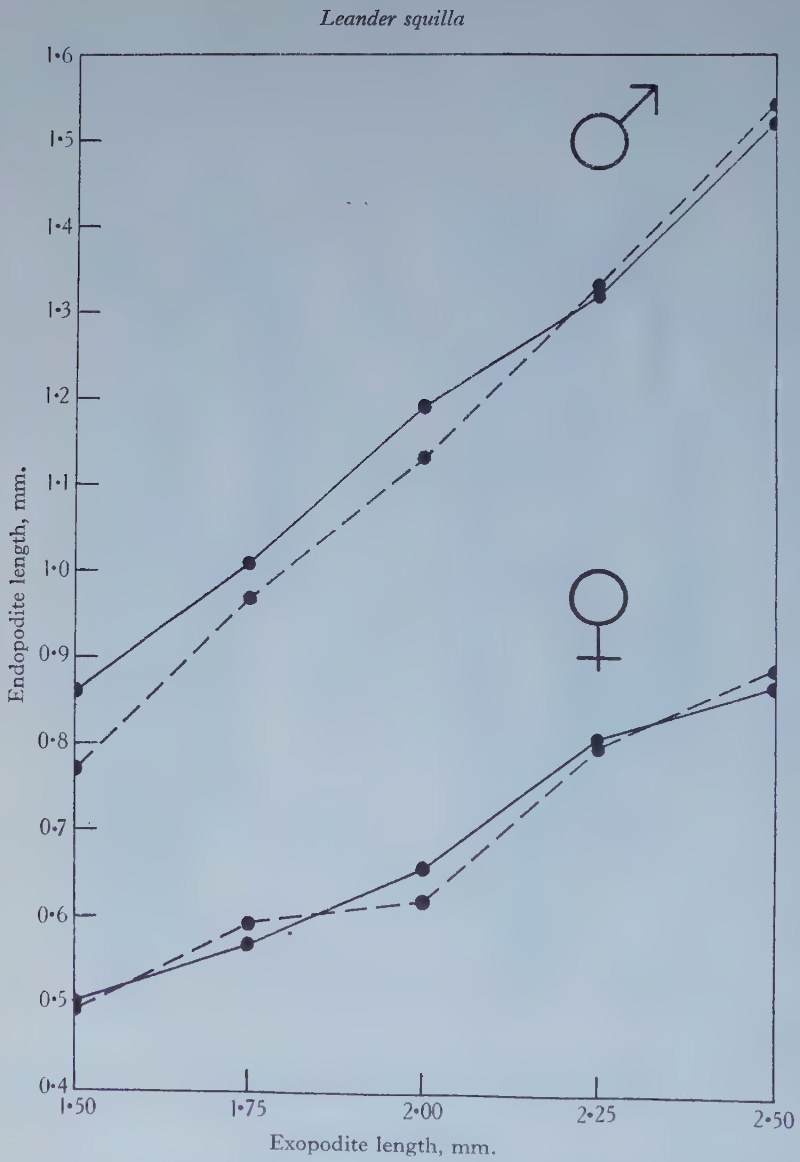
Text-fig. 1. Distal regions of pleopods on left side of male *Leander xiphias*, viewed from behind. 1 = 1st pleopod; 2 = 2nd pleopod. *Cop. St.* = copulatory style. *End.* = endopodite. *Ex.* = exopodite. *Ret.* = retinaculum. × 25.

graphs. In all, 1057 animals were used: 270 normal and 297 parasitized specimens of *L. xiphias*, and 245 normal and 245 parasitized specimens of *L. squilla* (see Text-figs. 2, 3).

The measurements indicated that there was a considerable degree of variability between individuals, but none the less, as is shown by the graphs, no significant difference between parasitized and unparasitized males. The same conclusion emerges from a study of the relative sizes of the copulatory style and endopodite of the second pleopod: parasitized males show no reduction of this organ. There is a



Text-fig. 2. Graph showing endopodite length plotted against exopodite length for the first pleopod of *Leander xiphias*. Full lines = normal animals. Broken lines = parasitized animals. Each point represents mean of many individuals, grouped into exopodite size groups of 0·25 mm. interval. (Total number of animals examined = 567.)



Text-fig. 3. As Text-fig. 2, but for *Leander squilla*. (Total number of animals examined = 490.)[1]

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[1] The correlation tables used in the construction of the graphs have been deposited at the British Museum.

reat deal of variability in the length and degree of curvature of the hooks on the ndopodite of the first pleopod, but here again there is no difference between arasitized and unparasitized males. In fact, I am forced to conclude that in these vo species of *Leander*, parasitism by *Bopyrus* does not cause sex reversal.[1]

Extreme reduction of the gonad is shown by both sexes when parasitized. The ontrast is most striking in the breeding season, when mature females of both pecies have ovaries of approximately 12 mm. length and 5 mm. width at the widest art, while a parasitized female of the same size may have an ovary of 4 mm. length nd 2 mm. width or even smaller. Sections of such an ovary show that the oocytes ever store yolk, remaining through the breeding season in the condition of those f an immature normal female. The oocytes reach a maximum size of 0·12 mm., hereas a normal ovarian egg with yolk storage at its maximum has a diameter of ·45 mm. (see Pl. I, figs. 1, 2). Parasitized females are completely sterile.[2]

The effect on the testis of *Leander* is not so precisely definable. There is con-iderable reduction in the rate of sperm production, as evidenced by the small umber of sperm stored in the vasa deferentia, and, more directly, by the few ivisions in spermatogonia and spermatocytes. This is more marked in *Leander iphias* than in *L. squilla*. It is possible that in some instances the parasitized males re capable of fertilizing the females, but I have no data on this point.

At no time are oocytes to be found in the testes of these two species of *Leander*, hether the animals are parasitized or not. Neither do they develop in the testes f males from which the parasites have been removed up to a maximum time of 37 days, long before which time the gonad has been restored to active sperm roduction. In these features the *Leander* testes show a marked contrast to the estes of *Upogebia* as described by Tucker (1930).

Discussion

The complete absence of sex reversal in these two species of *Leander*, when ther Macruran Decapods show the phenomenon so strikingly (if Giard's inter-retation be correct, even other species of *Leander*), is a fact of some significance n the analysis of the general problem of "castration parasitaire". In what causal ay does the situation in *Leander* differ from that of *Upogebia*? It is very unlikely hat the nutritional drainage effected by the Epicarid is less in *Leander*. The great eduction of the gonad is a criterion of just how heavy this drainage is, and, were the difference to originate from this cause, one would have expected that at least some effect would be shown by the secondary sex characters. Furthermore, it must be remembered that in those cases where sex reversal occurs, the authors have frequently remarked how little correlation there is between the state of the gonad and that of the secondary sex characters (Courrier, 1921; Tucker, 1930).

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[1] By the kindness of the authorities at the British Museum and at the Marine Biological Laboratory, lymouth, I have been able to examine a few further species of prawns carrying Epicarid parasites.) *Leander serratus* (Pennant), Epicarid *Bopyrus squillarum* Latreille. Out of about eighty specimens, hirty were males and in no case was there any sign of sex reversal. (*b*) *Pandalina brevirostris* (Rathke), picarid *Pleurocrypta cluthae* T. Scott. Out of seven specimens, two were males showing no signs f sex reversal. (*c*) Nineteen specimens of *Processa canaliculata* Leach = *Nika edulis* Risso, *Hippolyte* pp. and *Spirontocaris* spp. parasitized by *Phryxus* spp. or *Bopyrus* spp., were all females. In no case as there any trace of the copulatory style, and thus it seems unlikely that any were sex-reversed ales. However, the fact of not finding a single male in this material is puzzling.

[2] This is not the case in *Pandalina brevirostris*, where I have seen a parasitized female carrying few eggs in the incubatory chamber. If the parasite dies, females of *Leander serratus* can recover ompletely and bear a full complement of eggs in the incubatory chamber.

The fundamental difference between the two host-parasite relationships would appear to lie, therefore, not in any variable factors brought in by the parasite, but in those peculiar to the host species. Tucker has pointed out that there is a graded series of types showing sex reversal: (*a*) as in *Inachus* (Smith, 1906) where oocytes develop in the testes of males from which the parasite *Sacculina* has been removed; (*b*) as in *Eupagurus* (Potts, 1906) where oocytes develop in the testes while the parasite *Peltogaster* is *in situ*; and (*c*) as in *Upogebia* (Tucker, 1930) where oocytes develop in the testes of many males quite independent of parasitic attack, their number, however, augmenting as a result of parasitism by *Gyge*. This series may well be considered to show an increasing tendency towards intersexuality in the normal sex determination, a situation which can arise, as Goldschmidt (1931) has shown in the interracial crosses of *Lymantria*, from a greater or lesser degree of unbalance in the relative strengths of the sex-determining genes. The parasite then merely accentuates the latent possibility of sex reversal.

The fact that oocytes develop in the testes of males which are parasitized indicates the level at which the parasite acts. Its effects on the secondary sex characters are not due to an alteration in the production of some specific hormone by the testes; they are due to alteration in the production of the fundamental sex-determining substance or substances, as Smith emphasized. In an animal where sex is but weakly determined, the parasite can produce sex reversal, but in *Leander xiphias* and *L. squilla*, where there is apparently precise and complete "sex separation", *Bopyrus* cannot succeed in tipping the scales from male to female.

II. THE EFFECTS OF CASTRATION ON THE CYCLICAL BREEDING CHARACTERS OF THE FEMALES

Sollaud (1923), in a work on the embryology of *Palaemon* (*Leander*), has described a series of modifications which arise in the abdominal region of breeding females and result in the formation of an incubatory chamber for carrying the eggs during the early developmental stages. He has suggested that the differentiation of these breeding characters is directly dependent on the state of the ovary at maturity.

Observations

I will first describe the nature of these modifications. To form the roof of the incubatory chamber, the first three abdominal sternites become greatly expanded laterally, thereby separating the bases of the first three pleopods. The sides are formed from the enlarged pleura of the first three abdominal segments, the second pleuron overlapping both the one in front and the one behind. The precoxae of the first three pairs of pleopods become elongated, while the external margins of the asipodites of these pleopods become expanded posteriorly forming the “lames xternes” of Sollaud. These modifications Sollaud describes as the “caractères econdaires tardifs” and he states that they are definitive structures appearing at he first breeding moult and persisting afterwards. However, I have kept some emales of *Leander squilla* from the breeding period (in Naples this extends from ebruary to May) into the out-of-season condition and can thus say that these haracters are really cyclical in their development. It would be surprising if this ere not so, for during the winter one can only find unmodified females, and this pecies is certainly capable of breeding for more than one season.

In addition to these characters there are specially developed hair tracts con-erned in the formation of the incubatory chamber. First, there are the hairs which ear the eggs when these are laid. These are the “soies basales internes” of Sollaud, tout and unbranched, arising from the internal ridge of the basipodites of the first hree pairs of pleopods, one each from the coxopodites of the first four pairs of leopods, and a small tuft springing from the base of the basipodite of the fourth air of pleopods. The other hair tracts have a protective function. On the first hree pairs of pleopods a row of long barbed hairs arise from the internal surfaces f the “lames externes”, pointing inwards and posteriorly (“soies basales postéro-xternes” of Sollaud). From the anterior borders of the basipodites of the first our pairs of pleopods spring a row of hairs, much better developed in the first pair f pleopods than in the others (“soies basales antérieures” of Sollaud). Finally, here is a pair of hair rows, also long and barbed, borne by the internal margins of he fourth pair of pleopods, which point inwards and mat together in the mid-line hus forming the posterior limit of the incubatory chamber (see Text-figs. 4, 5). These hair tracts are the “caractères sexuels périodiques” of Sollaud, though, as we ave seen, there is really no justification for this distinction.[1]

These breeding characters all arise at the moult before the deposition of the ggs, and, since it is very rare to find a female with these characters developed, yet ith the eggs still in the ovary, we must suppose that egg deposition occurs im-ediately after the moult. If there is no further egg-laying after the first batch of oung has hatched, at the next moult these breeding characters are lost; sometimes, owever, there is a further egg-laying, and then they are retained at the moult.

Large parasitized females of *L. xiphias* and *L. squilla* at the breeding season orm none of these characters, neither do they carry any eggs. This is not a result f an inhibition of moulting; as pointed out on p. 169, the moulting of parasitized emales occurs more frequently than that of normal females. It implies, as Sollaud as suggested, a correlation between the state of the gonad and the formation of the reeding characters.

In an attempt to find out whether this was really the case, some large females ere castrated with X-rays. Females of *L. xiphias* were chosen measuring 6 cm. r more from the tip of the rostrum to the tail, and of *L. squilla* measuring 4·5 cm. In he breeding season all unparasitized females of these sizes develop the breeding characters and carry eggs. I found that the maturation of the oocytes could be entirely or almost entirely inhibited by a single dose of 6800 r. units. This was obtained by an exposure of 20 min. at 12 cm. distance from the anti-cathode, using 180 kV. and 10 mA. with the instrument at my disposal. Pl. I, figs. 3, 4, shows sections of ovaries of irradiated animals after a lapse of 6 weeks. Mortality o

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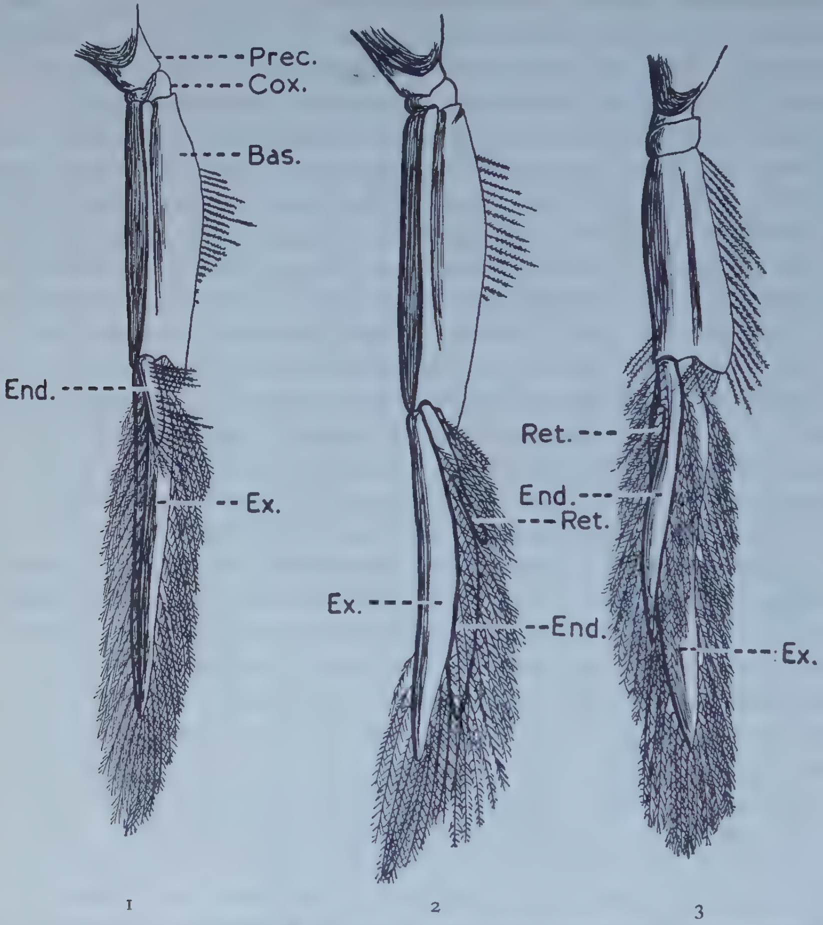
[1] A further character developed by the breeding females of *Leander* consists of large numbers of hite chromatophores around the incubatory chamber (see Knowles & Callan, 1940).



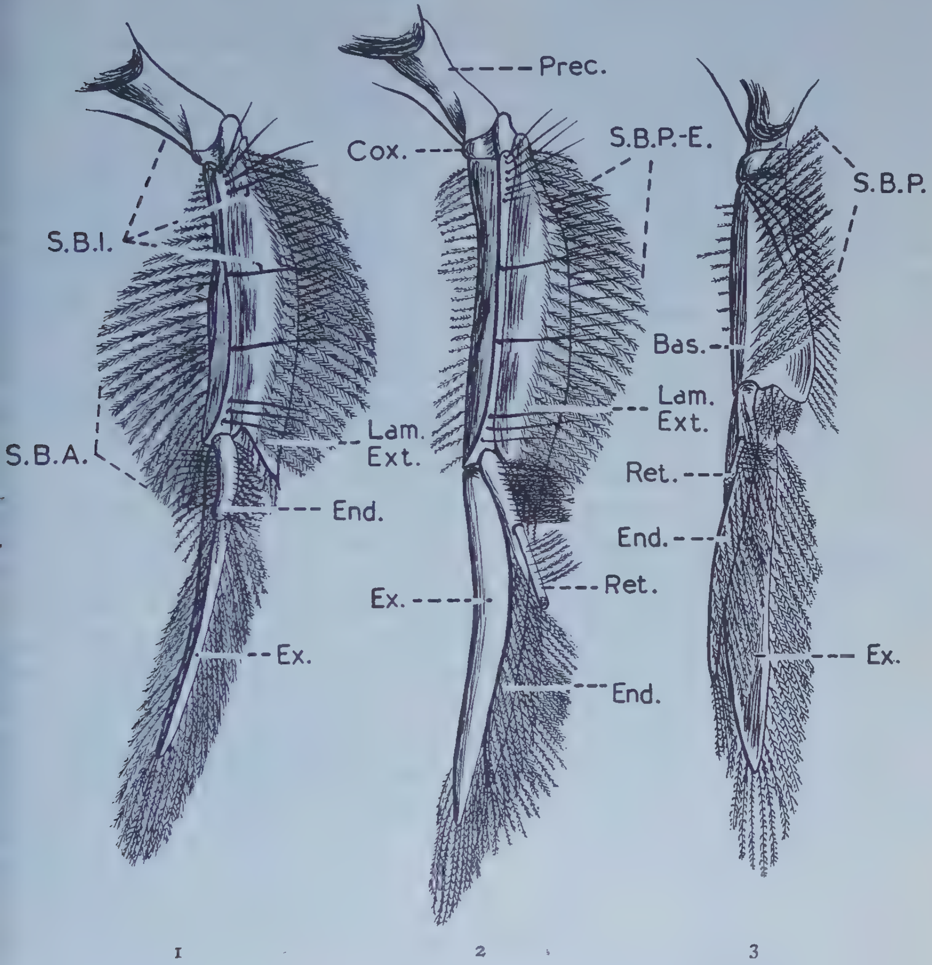
Text-fig. 4. Pleopods on the right side of female *Leander xiphias* out of the breeding season, viewed from the left. 1 = 1st pleopod; 2 = 2nd or 3rd pleopod; 3 = 4th pleopod. *Bas.* = basipodite. *Cox* = coxopodite. *End.* = endopodite. *Ex.* = exopodite. *Prec.* = precoxa. *Ret.* = retinaculum. × 10.

animals subsequent to irradiation was heavy, occurring mainly at moults, but in spite of this about thirty significant results were obtained for each species. The experimental animals were kept with males through the breeding season, during which time they moulted, but formed none of the breeding characters, which were developed by the controls.

I wish here to express my thanks to Prof. F. Pentimalli and the staff of the Cancer Institute in Naples for allowing me to use their X-ray apparatus, and for much personal assistance.

Discussion

These observations on the formation of cyclical breeding characters parallel ose of two other authors. Haemmerli-Boveri (1926) has described the develop-ent of the brood sac in *Asellus*, showing that it appears in a cyclical manner in



ext-fig. 5. Pleopods on the right side of female *Leander xiphias* in the breeding season, viewed from e left. 1 = 1st pleopod; 2 = 2nd or 3rd pleopod; 3 = 4th pleopod. *Bas.* = basipodite. *Cox.* = coxo-dite. *End.* = endopodite. *Ex.* = exopodite. *Lam. Ext.* = "lame exterme". *Prec.* = precoxa. *Ret.* = tinaculum. *S.B.A.* = "soies basales antérieures". *S.B.I.* = "soies basales internes". *S.B.P.* = soies basales postérieures". *S.B.P.-E.* = "soies basales postéro-externes". × 10.

dult females in correlation with the state of the ovary at the moult. On castration ith radium radiation, the brood-sac regressed through the subsequent moults and mained so for the rest of the animal's life.

The experiments of Le Roux (1931 *a*, *b*) are also completely in agreement. She und that in the normal development of *Gammarus*, at the ninth moult oostegites appear in the females and at the fourteenth moult these bear ovigerous hairs. Vitellogenesis and the formation of the ovigerous hairs coincides in time. However, when the females are parasitized by the Acanthocephalan larva *Polymorphus minutus*, although the animals moult the ovigerous hairs do not appear. Using radium bromide needles, she was able to check the maturation of the ovaries for a time. A corresponding delay in the appearance of the ovigerous hairs occurred, in spite of moults in the interim period, but when vitellogenesis of the oocytes recommenced there was a precisely correlated formation of the ovigerous hairs. She found, on the other hand, that the development of the oostegites went on quite independently.

The natural deduction to be drawn from this work is that the differentiation of such breeding characters in the females studied is directly dependent on stimulation from substances elaborated in the ovary and liberated in the blood. The close nature of the correlation is obvious, but unfortunately the experimental conditions of X-ray and radium castration are not critical. It may always be argued that the effects on the somatic tissues is a direct one, and the lowered viability of the experimental animals gives weight to this criticism. Further, there is the possibility that gonad and secondary sex characters are together reacting to some common third factor, general metabolic level, for example, or nutrition, as Avel (1929) found to be the governing factor in the development of the clitellum of *Lumbricus*. It is unfortunate that surgical castration of a crustacean is impracticable. In any case, however, castration experiments must be supplemented by injections of gonad extracts or gonad implantations before the presence of a hormone can be proved. The analogy between the development of the breeding characters of *Leander* and the preparation of a female mammal by luteal hormone for the birth of young is none the less striking, even though the mechanism of co-ordination may be entirely different in the two cases.

Finally, a point to notice is that the effect of *Bopyrus* on the metabolism of the host does not balance the inactivity of the ovary in the parasitized animal. This host-parasite relationship clearly does not follow the lines of the more familiar cases of "castration parasitaire".

SUMMARY

1. Contrary to the assertion of Giard that *Leander* males when parasitized by *Bopyrus* undergo sex reversal, *Leander xiphias* and *L. squilla* show no modifications of the secondary sex characters, while the testes, although reduced in size, never produce oocytes. This is probably true also of *L. serratus*.

2. The power of a parasite to induce sex reversal is probably limited by the degree of sex separation of the host species. The species which show sex reversal ("castration parasitaire") are thus considered to possess a less complete sex-separating mechanism than those, such as *Leander*, which are not reversed by parasites.

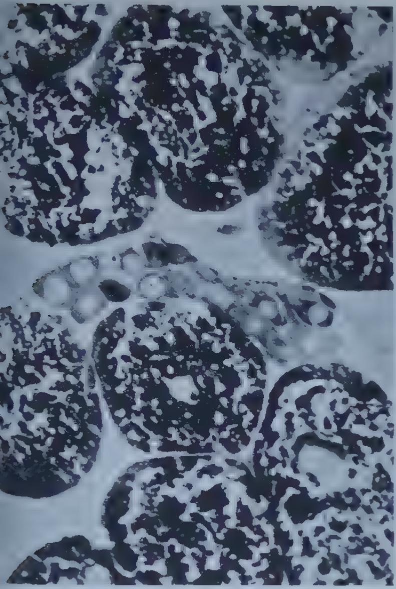


Fig. 1.

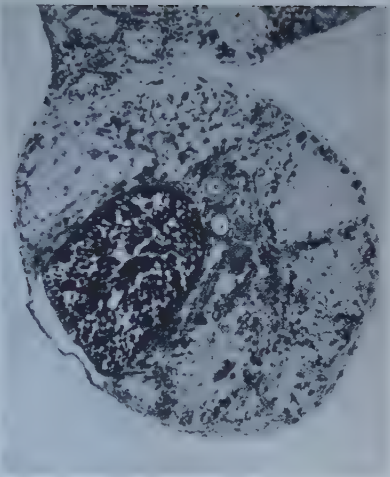


Fig. 3.

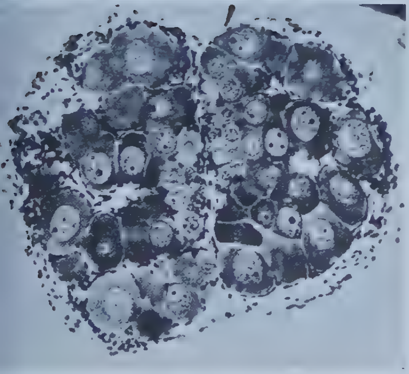


Fig. 2.

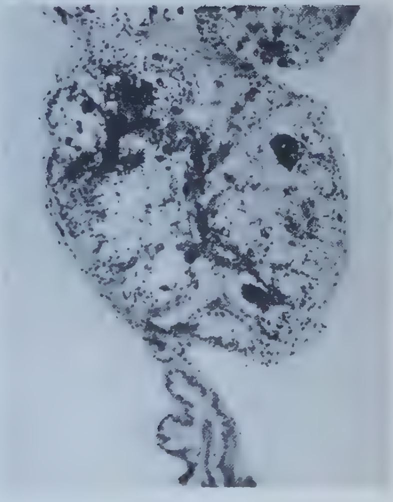


Fig. 4.

CALLAN—THE EFFECTS OF CASTRATION BY PARASITES AND X-RAYS ON THE SECONDARY SEX CHARACTERS OF PRAWNS (*LEANDER* SPP.) (pp. 168–179)

3. Breeding females of *Leander* develop an incubatory chamber under the domen, involving many structural changes.

4. These "breeding characters" are not formed by parasitized females, nor by males castrated by X-rays.

5. It is possible that there is a female sex hormone in Crustacea, but the idence is not yet conclusive.

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EXPLANATION OF PLATE I

ections of ovaries of *Leander xiphias*, Bouin-Sea water, Iron Haematoxylin. (All × 60 diameters.)) Small region of section of ovary fixed in the breeding season before egg deposition, showing cytes filled with yolk, and a reserve of immature oocytes. (2) Section of whole ovary of parasitized male in the breeding season, showing entire absence of yolk in the oocytes. (3) Half of section of ary fixed in the breeding season, six weeks after X-ray treatment. One oocyte has escaped destruc- on and accumulated yolk. A few residual immature oocytes appear unharmed. The remainder of e oocytes were destroyed during vitellogenesis. (4) As (3), but castration by X-rays complete. o oocytes present.

LOCAL AND GENERAL FACTORS IN THE DEVELOPMENT OF "PATTERN" IN *RHODNIUS PROLIXUS* (HEMIPTERA)

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(With One Plate and Thirteen Text-figures)

Most studies on morphogenesis have been made upon composite structures, such as eyes or appendages, whose form is controlled by the co-operation of a grea number of cells. In insects, however, the tiny fragment of cuticle laid down by a singl cell may possess morphological characters controlled by the activities of that cel alone. The cuticle of insects provides, therefore, an unusually simple medium fo the study of morphogenesis and the nature of those processes which "determine" the activity of a given cell.

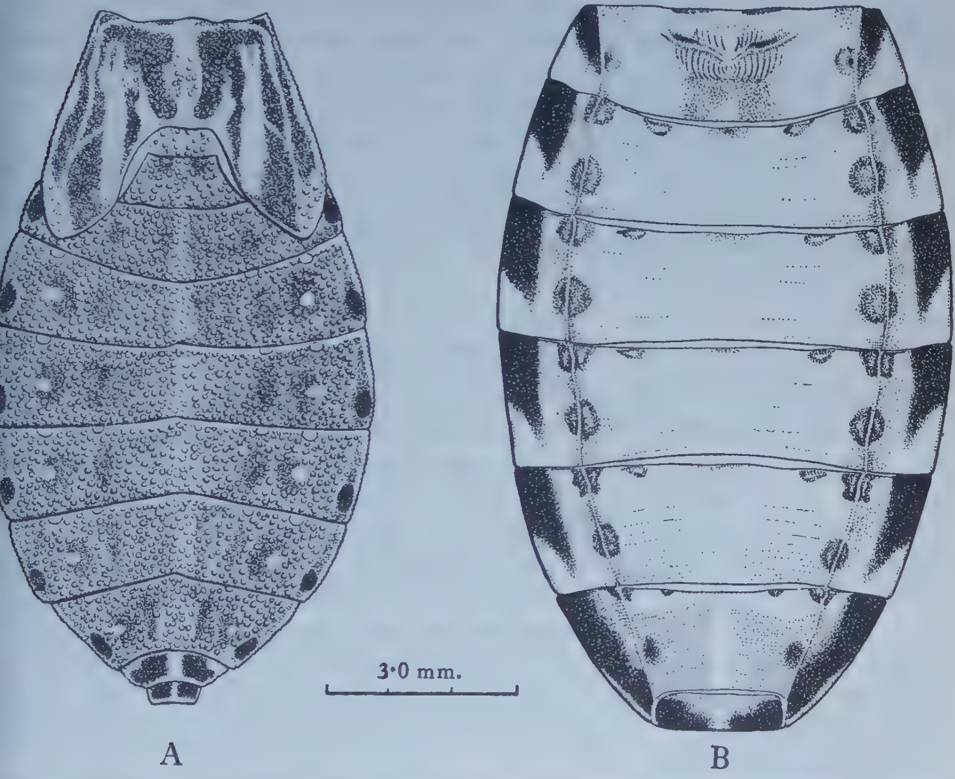
As material for the present work the epidermis of the abdomen in the blood-sucking bug *Rhodnius* has been employed. This consists of a single layer of cells which lays down an extracellular cuticle. The cuticle has a "pattern", since it varie in structure and in coloration in different regions. As we shall see, the individua cells in each region are "determined" for the formation of different elements in this pattern. Certain elements in the pattern, however, are capable of regeneration In other words, certain of the cells retain potencies which are normally suppressed The material therefore affords an opportunity of studying the process by which th realization of such latent potencies is brought about. Finally, the pattern show well marked changes at metamorphosis, and these changes are controlled b hormones (Wigglesworth, 1934, 1936). The material can therefore be used to stud the mode of action of these general humoral factors upon local changes in development; to study, that is, the interaction between the circulating hormones on th one hand and the competent tissue on the other.

DESCRIPTION OF THE ABDOMINAL CUTICLE IN *RHODNIUS*

Cuticle of the nymphal stages

The structure and chemistry of the cuticle in *Rhodnius* has been described in a earlier paper (Wigglesworth, 1933); but in order to make what follows intelligibl it will be necessary to describe the structure again from a slightly different point o view.

There are five nymphal stages in *Rhodnius*, throughout which the pattern of the bdomen changes comparatively little. Text-fig. 1 A shows the dorsal surface of a th stage nymph. Nine segments are visible from above. These are separated by ransverse folds in the cuticle—the *intersegmental membranes*. The cuticle is chiefly omposed of a relatively thick endocuticle which is exceedingly elastic and enables he insect to ingest up to 12 times its own weight of blood at a single meal. This ndocuticle is overlaid by a very thin epicuticle thrown into *stellate folds* (Pl. I, g. 1) which become partially smoothed out as the endocuticle stretches. The picuticle is diffusely pigmented with melanin.



ext-fig. 1. A, dorsal view of meso- and metathorax and abdomen of 5th stage nymph of *Rhodnius*. B, dorsal view of abdomen of adult female.

Scattered over the surface are *bristles*, which arise from little mounds of smooth ticle termed *plaques*. In the region of each plaque an exocuticle is present, the ter part of the cuticle being impregnated with the so-called "cuticulin", and the icuticle is without folds (Pl. I, fig. 1). Around the bristles the cuticle is therefore extensible. The bristles and the plaques are unpigmented and thus appear as pale ints over the surface of the abdomen. Along its borders the abdomen bears a uble or treble row of *marginal plaques and bristles* set very close together (Text-fig. A).

On either side of each abdominal segment there are three *muscle insertions* in the cuticle, two at its anterior margin and one in the middle, which appear as round or oval unpigmented areas. And near the posterior angle of each segment there is an oval *pigment spot* in which the outer layers of the cuticle are deeply impregnated with melanin.

A final element in the pattern is produced by local differences in the numbers of plaques. They are absent from the intersegmental membranes and from a narrow zone on either side of these; there is a zone just internal to the marginal plaques and the pigment spot which is free from them; the areas overlying and immediately around the muscle insertions are devoid of them; and there is always a patch half way between the central muscle insertion and the mid-line of the body which contains a campaniform sensillum and is relatively free from plaques. These areas in which the plaques are scanty are rather more deeply pigmented.

Beneath the cuticle is a single layer of epidermal cells by which it is laid down during moulting. Pl. I, fig. 3, shows the relation between these cells and the plaques. In addition there are numerous little dermal glands opening through the cuticle, and oenocytes scattered below the epidermal cells (Wigglesworth, 1933); with these we shall not be concerned.

Cuticle of the adult

Text-fig. 1 B shows the corresponding pattern in the adult *Rhodnius*. The segments are separated as before by *intersegmental membranes*, but the structure of each segment is markedly changed. Except at the intersegmental membranes, the outer half of the cuticle is impregnated with "cuticulin" to form an exocuticle. It is therefore inextensible. The epicuticle is less markedly folded. The *type of folding* varies in different regions but over most of the surface it consists of little parallel transverse ridges, with minute branched folds running from them (Pl. I, fig. 2). The plaques have disappeared and, except on the 7th and to a less extent on the 6th segments, *bristles* are very scanty over the central area of the tergites. The lateral region of each segment, on the other hand, bears abundant bristles, without plaques, and the double row of *marginal bristles* is again conspicuous, though their form is changed (Text-fig. 6 C).

The *muscle insertions* are present as in the nymph, though they are now covered by pigmented cuticle contrasting with the cuticle elsewhere which is unpigmented. A pigment spot is again conspicuous in each segment; but it lies now at the anterior angle and reaches more than half way across the segment, tapering to end in a point behind.

Somewhat internal to the pigment spot, and passing through the two outer muscle insertions, a line runs parallel with the margin which will be termed the *hinge line*. As previously shown, the exocuticle is wanting along this line (see Text-fig. 8 B, of the next paper (Wigglesworth, 1940)); the cuticle is therefore enabled to bend here after the ingestion of a large meal (Wigglesworth, 1933). In mounted cuticles the hinge line appears colourless against the grey or amber-coloured cuticle n either side of it. On segments 1 and 2 the cuticle in the central region is pigmented nd thrown into little curved parallel ridges.

On the ventral surface of the abdomen abundant bristles, without plaques, are etained. It is not necessary to describe the local differences these show. But close o the lateral margin on the ventral surface there is a longitudinal band of cuticle n which the exocuticle is wanting and the epicuticle is thrown into elaborate ongitudinal folds. This is termed the *lateral pleat*; as already described (Wigglesvorth, 1933) it is highly elastic and, working in conjunction with the hinge line, it nables the abdomen to expand and receive the enormous meals of blood.

HE PART PLAYED BY INDIVIDUAL CELLS IN THE DETERMINATION OF NYMPHAL PATTERN; THE EFFECT OF WOUND HEALING ON PATTERN

In an earlier paper it was shown that during the healing of wounds in the pidermis of *Rhodnius* the cells around the injury may migrate inwards, without ividing, for considerable distances to make good the defect. In the healing of burns his migration of existing cells is not so great as it is when the tissues are cut; after burn the dead epidermis is replaced by the cells immediately outside the injury ividing repeatedly and spreading inwards until continuity is restored (Wigglesvorth, 1937).

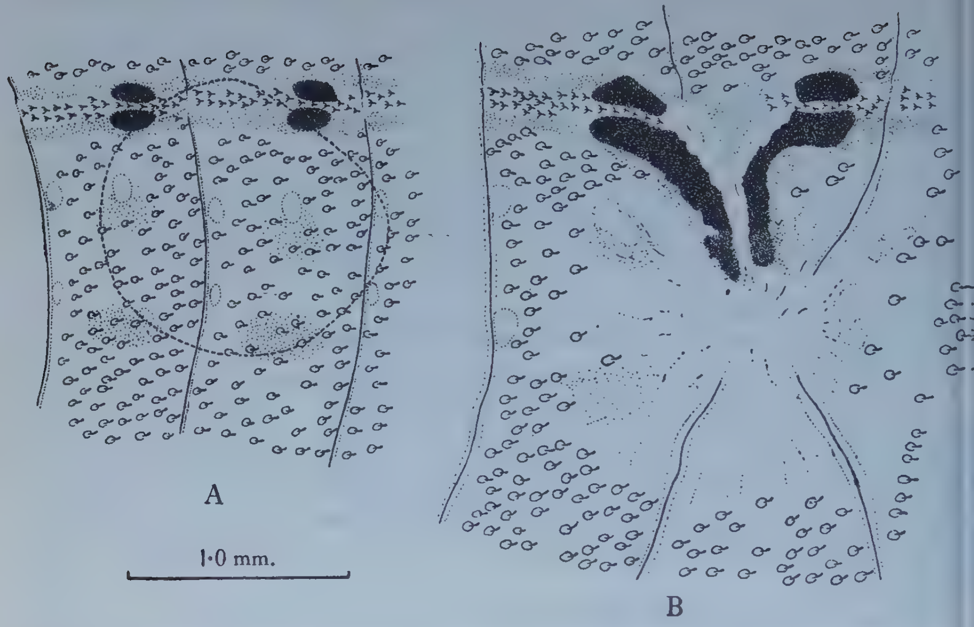
It was interesting to see whether these cells, as they multiply and spread inwards, etain their original capacities to lay down particular elements in the pattern of the uticle; or whether their potencies are lost and they become "de-differentiated"; r whether they can develop other characters determined by the region into which hey migrate.

This question was investigated by burning areas of variable position and extent n the dorsal cuticle of nymphs in the second or third instars by means of melted araffin wax applied by a heated wire. The burns were inflicted one day after feeding. he insects were then allowed to moult, and the effects on the abdominal pattern oted in later instars.

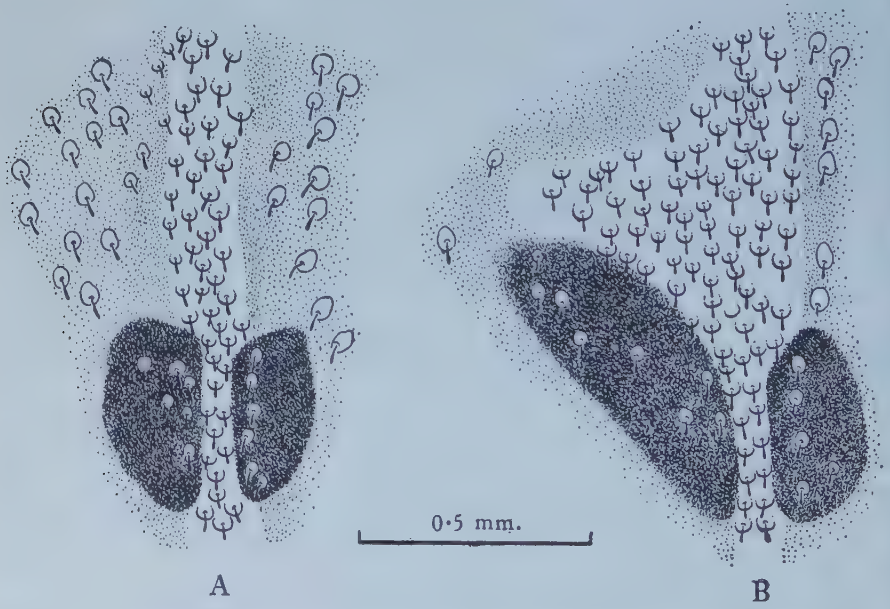
Text-fig. 2 represents a typical example of the results obtained. In Text-fig. 2 A he approximate extent over which the epidermis in the 3rd stage nymph was illed is indicated by the broken line. In Text-fig. 2 B the resulting pattern in the th stage nymph is shown. It is evident that as the cells which were destined to orm pigmented cuticle divided and migrated towards the centre of the wound they rought with them the capacity to lay down cuticle of this type; and the cells from he intersegmental regions behaved similarly.

Text-fig. 3 B shows another example. The burn in the 3rd stage nymph exended exactly into the line of marginal plaques. Consequently, in the 5th stage ymph, the marginal plaques as well as the pigment spot have spread far inwards. ext-fig. 3 A shows the corresponding region in a normal 5th stage nymph for omparison. Other experiments have shown that when the edge of the burn extends nto regions normally devoid of plaques these regions become similarly enlarged.

It is evident that the cells which form a given element in the pattern retain their roperty of forming this element in spite of repeated division and migration.



Text-fig. 2. A, lateral region of the dorsal surface of two segments in a 3rd instar nymph, showing the double row of marginal plaques above, and beyond this a small piece of the ventral surface. The broken line shows the extent of the epidermis killed by burning. B, the corresponding region in the 4th instar after healing and moulting.

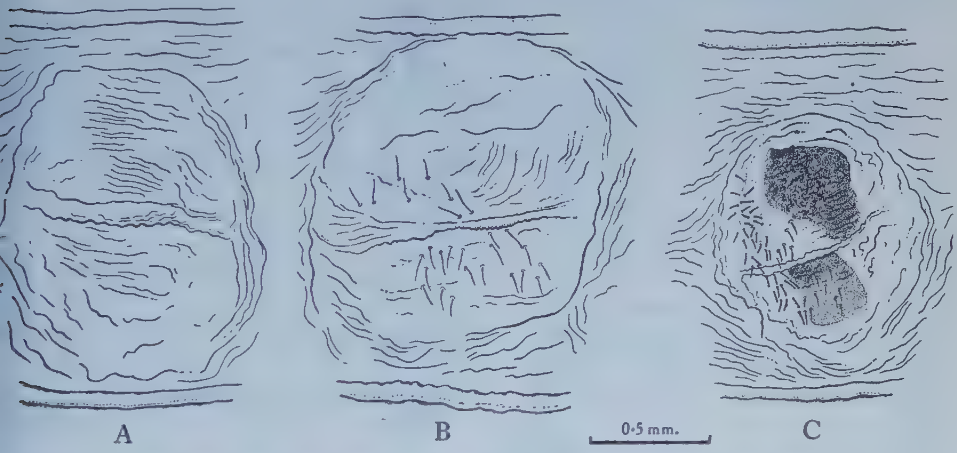


Text-fig. 3. A, marginal plaques and adjacent tergal and sternal black spots in normal 5th instar nymph. B, corresponding region in a 5th instar nymph burned on the tergites in the 3rd instar, showing extension of the marginal plaques and of the tergal black spot.

EFFECT OF WOUND HEALING IN THE NYMPH ON THE PATTERN IN THE ADULT

It seems probable that the imaginal pattern is already "determined" in the uite young nymph and only awaits the proper hormones to become manifest. or example, first stage nymphs can be caused to undergo a precocious metaorphosis, and assume adult characters when they moult, simply by decapitating iem (that is, depriving them of the secretion of their corpus allatum) at a particular ritical period in the moulting process (Wigglesworth, 1934).

This question has been further tested by excising small pieces of epidermis us cuticle (about 1–1·5 mm. square) from different regions of the abdomen of rd or 4th instar nymphs and implanting them into the central area of the back in



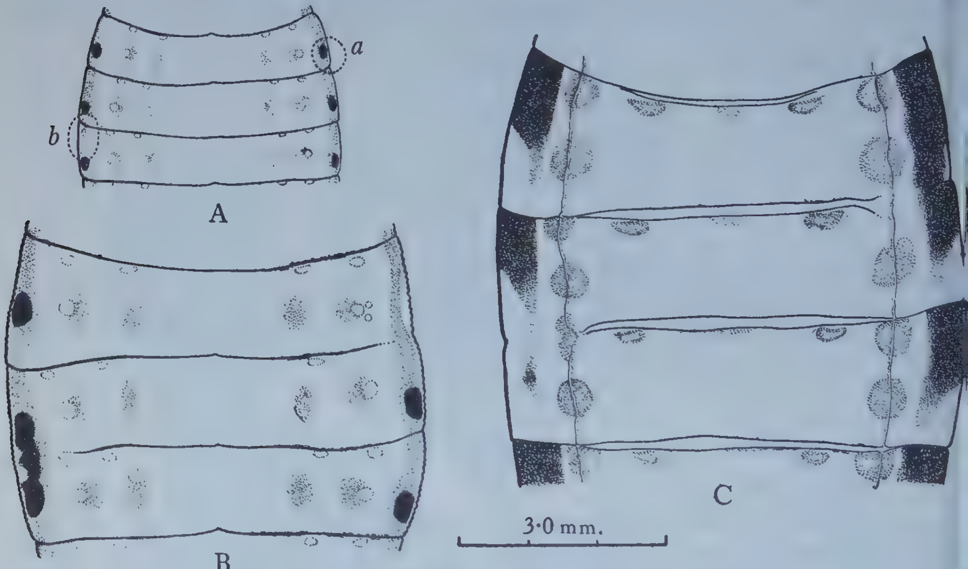
ext-fig. 4. Small pieces of cuticle from 4th instar nymphs implanted into the tergites of 5th instar ymphs and seen after moulting. Above and below in each case are the intersegmental membranes the adult host. A, mid-dorsal cuticle with intersegmental membrane implanted. B, ventral cuticle ith intersegmental membrane implanted. C, dorsal and lateral cuticle implanted, showing intergmental membrane, pigment spots and marginal spines.

th instar nymphs, after an excision of a suitable size had been made to receive nem; the margins of the wound being sealed with paraffin of low melting point. n each case the cuticle was removed from the 3rd or 4th instar nymph 24 hr. fter moulting; that is, at a stage when it was not capable of autonomous moulting. t was implanted into 5th stage nymphs a day or two after they had fed.

When the 5th stage nymphs moulted they became adults; and the implanted agments of cuticle were also caused to undergo metamorphosis and develop naginal characters. Then it was found that the implants developed imaginal cuticle f the type corresponding to the region from which they had been excised. Text-fig. shows three examples. In A, the cuticle was removed from the central area of the ack; in B, it came from the sternites, which are clothed with bristles in the adult; nd in C, it came from the side of the tergites, including the zone of marginal spines.

It appears therefore that although they are not manifested until metamorphosi the imaginal characters are determined at an early stage. That being so, if thi "determination" is again localized in the individual cells, it should be possible t produce changes in the imaginal pattern by injuries inflicted on the young nymph of the same kind as those described for the nymphal pattern in the last section.

The experiments were carried out in the same way except that the insects wer allowed to transform into adults. Text-fig. 5 A shows the position in which th epidermis was killed by burning in a 3rd stage nymph. The burn *a* complete destroyed the lateral black spot on segment 3. Consequently this spot was absen in the 5th stage nymph (Text-fig. 5 B). But the wound had been healed by mea of epidermal cells from the region of the prospective imaginal black spot. Consequently, in the adult (Text-fig. 5 C) the spot on the 3rd segment has becom continuous with that on the 4th.



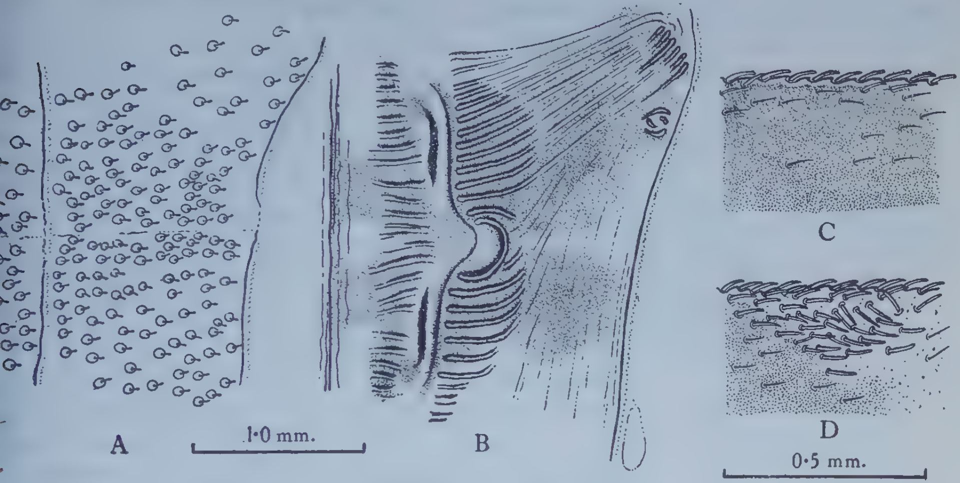
Text-fig. 5. A, third, fourth and fifth tergites of a normal 3rd instar nymph. The broken lines *a* and *b* show the regions burned. B, corresponding segments in the 5th instar nymph resulting C, corresponding segments in the adult resulting.

The burn *b*, on the other hand, extended from the margin of the black spot o segment 4 to the margin of that on segment 5, destroying the region of the prospective imaginal black spot. Consequently, in the 5th stage nymph (Text-fig. 5 B the black spots in this region have become united; whereas the black spot of th adult (Text-fig. 5 C) has been almost eliminated.

Text-fig. 6 illustrates two further examples. B shows the extension backward and outwards in the adult of the ridged cuticle on the 2nd segment. In the nymp this region of the abdomen does not differ from other parts. Hence, although th change is very obvious in the adult, there was no sign of it in the 5th stage nymp

Text-fig. 6 A). D shows the inward extension of marginal spines to replace a part of he imaginal black spot; and C shows the corresponding region in the normal insect.

Thus it is evident that the "imaginal determination", like the determination of ymphal characters, is a property of the individual cells already present in the ymph. When the cells divide and migrate, the imaginal properties migrate with hem; but these properties become manifest only under the influence of the appro-riate hormones at metamorphosis.



ext-fig. 6. A, part of mid-dorsal region of first and second segments in a 5th instar nymph which ad been burned on the right side in the 3rd instar. B, corresponding segments in the adult resulting. , marginal spines and part of one of the black spots of a normal adult. D, corresponding region owing marginal spines displacing the black spot in an adult that had been burned in the 3rd instar.

REGENERATION OF BRISTLES AND PLAQUES

The foregoing experiments prove that when all the cells possessing a given roperty (determined, that is, for a given purpose) are destroyed, there is no re-eneration of the structure they were destined to produce. Development of those ements in the abdominal pattern so far considered is possible only so long as some f the cells which would normally contribute to them remain.[1] This is not what is sually meant by regeneration.

In true regeneration, when cells which would normally produce a given structure e destroyed, adjacent cells become determined for this purpose and realize tentialities which would ordinarily remain permanently latent. This phenomenon shown by the bristles and plaques in the abdominal cuticle.

Text-fig. 7 shows the result in succeeding instars, of a burn in the general pidermis of a 2nd instar nymph. At the first moult after the burn, that is, in the d instar, the burned area is covered by cuticle with stellate folding of the usual nymphal type, but no new plaques arise—or at most two or three new ones at th extreme margin (Text-fig. 7 A). At the next moult, plaques have appeared all ove the cuticle with the exception of a small central region which is most remote from the margins of the burn (Text-fig. 7 B). At the next moult, that is, in the 5th instar new plaques have appeared in this central region also (Text-fig. 7 C).

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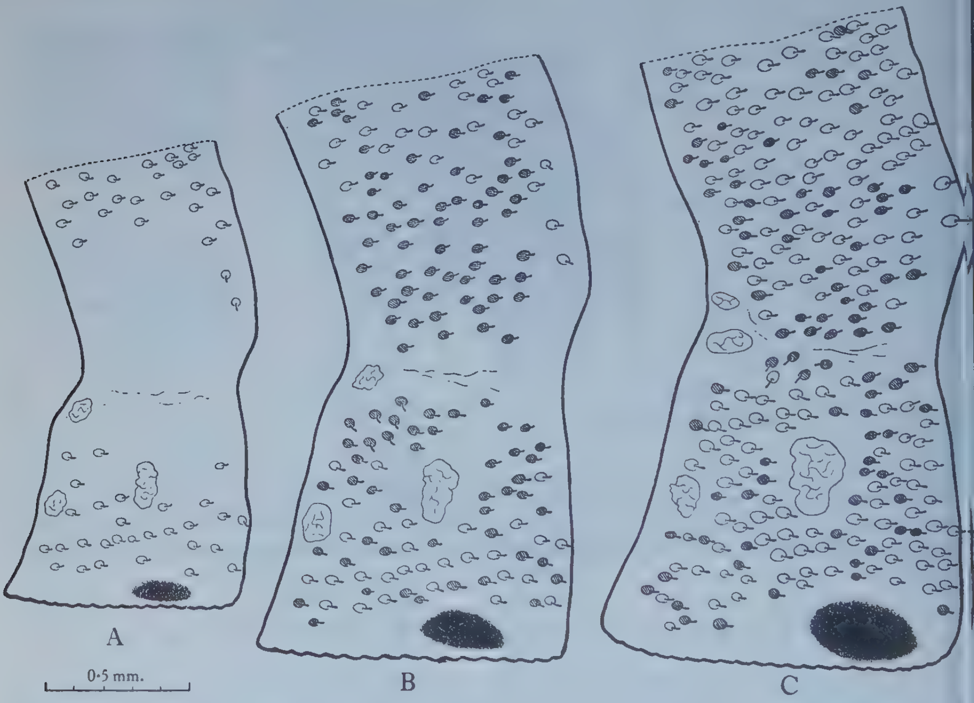
[1] From the experiments described it cannot be stated definitely that *no* cells outside the pigment ots, for example, retain the power of forming pigmented cuticle; but it seems probable that this is so.

Clearly, groups of cells at intervals from one another have become transforme into bristle- and plaque-forming units. The number and regular distribution o these units precludes the possibility that they have been produced by cells alread determined for this purpose. It is in fact evident that in appropriate circumstances cells which would normally contribute to the ordinary stellate intervening cuticle may become determined for the production of bristles or to contribute to the plaques around them.



Text-fig. 7. A, left half of third tergite of 3rd instar nymph after a burn in the 2nd instar. B, th same in the 4th instar. C, the same in the 5th instar. In B and C the newly formed plaques ar shaded, those appearing at previously existing sites plain.

When the burn is very extensive, a large central region may not only be devoid of plaques, but colourless and without stellate folding at the first moult after the burn. If the cuticle and epidermis are stained, the reason for this is apparent: the cells in this region are rather sparse and very irregular, with giant cells and other abnormalities previously described (Wigglesworth, 1937). Clearly, moulting has occurred before the epidermis in the central region has had time to reorganize itself properly. That is doubtless the reason why in Text-fig. 7 the central area of ne wound failed to develop plaques until the third moult after burning. This same gure shows another area (posterior to the centre of the burn) in which plaques failed appear. That is evidently because this area has been regenerated by cells from ne region of the intersegmental membrane where plaques are always absent.

It is worth noting that, with few exceptions, the regenerated plaques and ristles show the normal orientation. This raises a new problem which has not been rther investigated.

Before discussing the mechanism by which these regenerating plaques are etermined, we must consider the factors which control the appearance of plaques uring the normal course of development.

DETERMINATION OF BRISTLES AND PLAQUES DURING NORMAL DEVELOPMENT

We have seen that there are certain areas in the abdominal cuticle which are evoid of plaques (p. 182). These deficiencies are doubtless caused by local differnces in the properties of the cells in these regions, of the type described in foregoing ections. We have indeed seen that during the migration of cells in the healing of ounds, this property of not laying down plaques is retained by the cells from these gions. Over the greater part of the abdominal segments, however, the plaques e pretty evenly distributed. In this section we shall consider the factors which ontrol this distribution.

Throughout this section of the work the dorsal part of the third abdominal gment has been used as the standard object of study. During moulting the old uticle splits down the mid-line of the thorax, and the split extends into the first ree or four segments of the abdomen. The old cuticle as cast at moulting, or the ew cuticle formed, have been mounted, and the plaques in one entire half of this rgite, excluding the marginal plaques, have been traced by means of a drawing pparatus[1] and counted.

Text-fig. 8 shows a typical result obtained during the moulting of a 4th stage ymph. As pointed out in an earlier paper (Wigglesworth, 1933), the bristlerming cells persist from one instar to the next; so that most of the plaques arise the site of the previous plaques. But a certain number of new plaques are deeloped at each moult between the existing ones. In Text-fig. 8 B, the new plaques e shown shaded, the old plaques plain.

The first question that arises is whether these new plaques are "predetermined", whether their appearance is the result of the local conditions to which the cells e exposed—for we have seen that the potentiality to form plaques is apparently tained by all the ordinary epidermal cells.

It is evident in Text-fig. 8 that, apart from regions where the plaques are turally sparse, new plaques make their appearance at those points where the old aques were most widely separated. That suggests that the distance from existing aques may be the factor which leads to their determination. This question was sted by increasing artificially the mutual separation of the plaques by allowing the insect to ingest a full meal of blood and then immediately occluding the anu with paraffin wax so that the excess fluid could not be excreted in the usual way an the abdomen remained enormously stretched.

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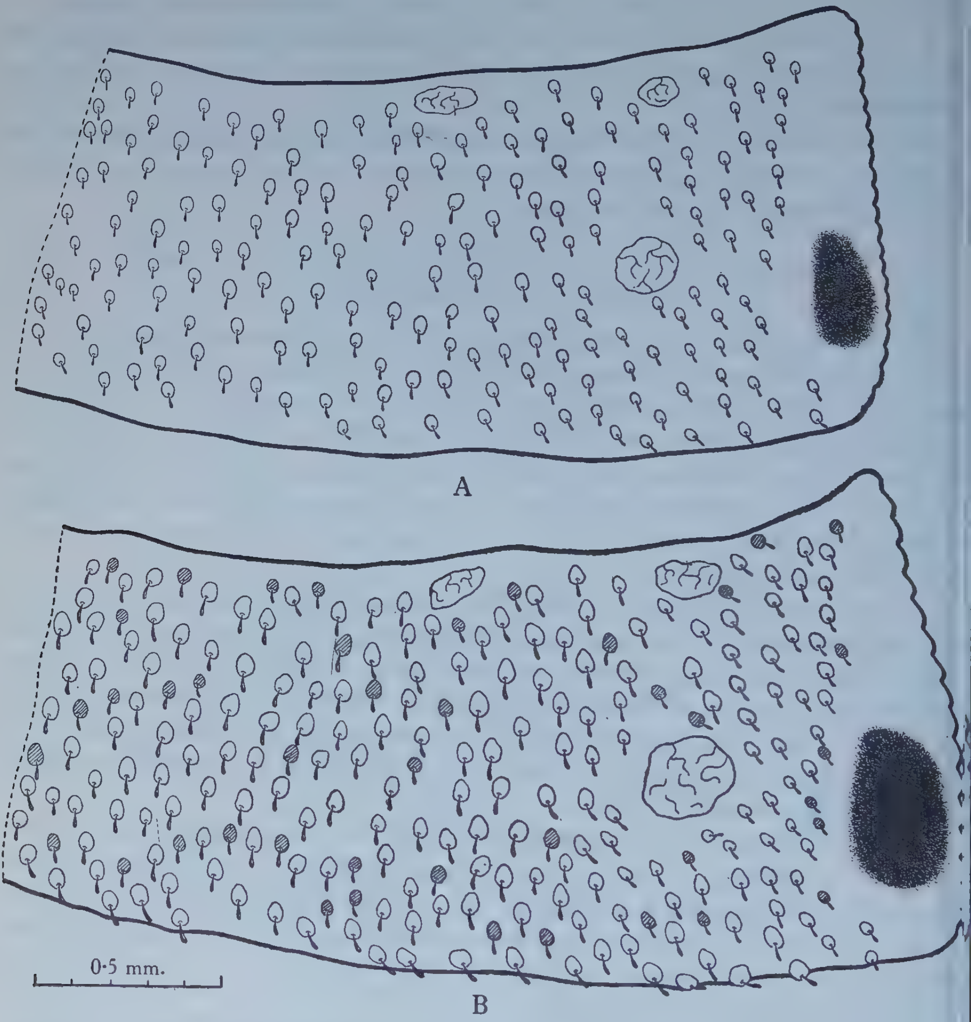
[1] The 'Moscon Macrograph' was used.

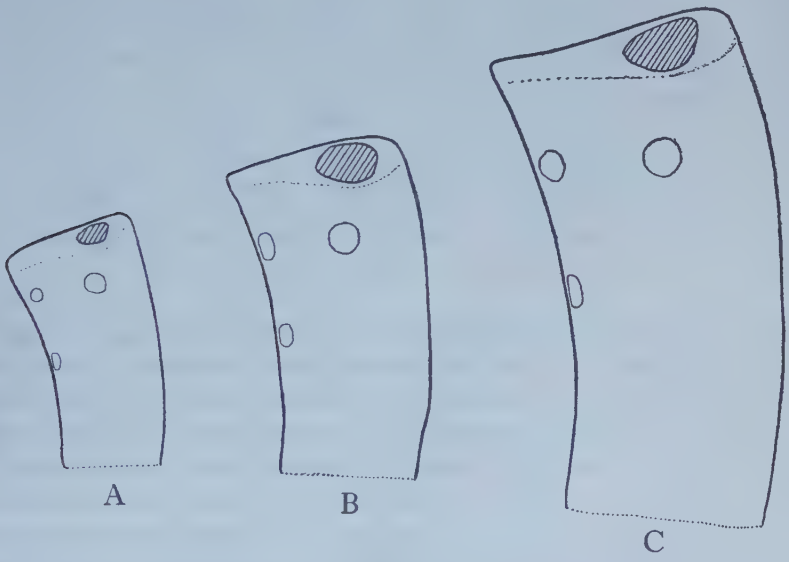


Text-fig. 8. A, right half of third tergite in normal 4th instar nymph. B, the same in 5th instar, th newly differentiated plaques shaded.

Table I shows the results obtained. The number of plaques on half of the thir segment of six 4th stage nymphs ranged from 174 to 217 with an average of 191 After moulting, in the normal insect, to become a 5th stage nymph, they range from 226 to 287 with an average of 244. The increase thus ranged from 19·3 t 32·1 % with a mean of 26·9 %. In five 4th stage nymphs in which the anus wa occluded, the number of plaques showed an average increase of 26·7 %.

able I. *The number of plaques on half the third abdominal tergite in 4th and 5th stage nymphs, showing the percentage increase when the 4th stage nymphs were normal, excessively stretched, or unfed*

	1	2	3	4	5	6	Mean
4th nymph (normal)	174	179	192	204	181	217	*191*
5th nymph	229	229	237	260	226	287	*244*
Increase %	31·5	27·9	23·4	27·4	19·3	32·1	*26·9*
4th nymph (stretched)	190	193	202	211	—	—	*199*
5th nymph	238	237	261	274	—	—	*252*
Increase %	25·2	22·8	29·2	29·8	—	—	*26·7*
4th nymph (unfed)	168	182	175	—	—	—	*175*
5th nymph	203	239	228	—	—	—	*223*
Increase %	20·8	31·3	30·3	—	—	—	*27·4*



ext-fig. 9. Right half of third tergite in 5th instar nymph. A, when 4th instar was unfed. B, when 4th instar was fed normally. C, when anus of 4th instar was occluded.

The converse experiment, of testing the effects of a minimal amount of separa-on between the plaques, was performed by inducing moulting in unfed 4th stage ymphs by decapitating them and joining them, in the manner already described Wigglesworth, 1934), to 4th stage nymphs in which the anterior part of the head as removed, leaving the corpus allatum intact, at six or seven days after feeding. n three such experiments the mean increase in the number of plaques was 27·4% Table I).

Text-fig. 9 shows the relative area of the half segment under consideration in hese three cases—unfed, normal and stretched. The area which the plaques occupy is in the ratio of about 5·4, 10·0, 21·7 in the three cases; and yet this difference ha no effect on the number of new plaques formed.

These results appear to disprove the influence of mutual separation in determining the appearance of new plaques. But the possibility remains that it is th degree of separation at the *preceding* moult which is important. This was tested b occluding the anus immediately after feeding in 3rd instar nymphs; allowing them to moult to the 4th instar; and then feeding these again without occlusion.

Table II. *The number of plaques on half the third abdominal tergite in 3rd, 4th an 5th stage nymphs, showing the percentage increase when the 3rd stage nymphs wer normal or excessively stretched*

	1	2	3	4	5	6	7	Mean
3rd nymph (normal)	126	112	143	119	139	128	147	*131*
4th nymph	186	172	204	181	217	202[1]	211[1]	*196*
Increase %	47·6	53·5	42·6	52·1	56·1	57·8	43·6	*50·4*
5th nymph	—	—	260	226	287	261	274	*261*
Increase %	—	—	27·4	19·3	32·1	29·2	29·8	*27·5*
3rd nymph (stretched)	132	130	137	128	138	—	—	*135*
4th nymph	195	195	190[1]	186[1]	206[1]	193	—	*194*
Increase %	48·5	50·0	38·7	45·3	49·3	—	—	*46·3*
5th nymph	265	274	286	271	289	292	—	*280*
Increase %	35·8	40·5	50·5	45·7	40·0	51·3	—	*43·8*

[1] These insects, in addition to the 3rd stage nymphs, were excessively stretched by occlusion of the anus.

Table II shows the results obtained. The normal 3rd stage nymph has an average of 131 plaques on the standard half segment. On moulting to the 4th stage the plaques show a mean increase of 50·4% (42·6–57·8). As in the case of the moulting 4th stage, as already described, occlusion of the anus in the 3rd stage nymph caused no increase in the number of plaques formed: 46·3% (38·7–50·0).

On the other hand, excessive stretching in the 3rd instar causes a great increase in the number of plaques formed when the 4th instar moults to become a 5th. The mean increase in plaques during this moult was 27·5% when the 3rd stage had been treated normally. But when the anus had been occluded in the 3rd stage this increase amounted to 43·8% (35·8–51·3) (Text-fig. 10).

In some of these experiments the anus was occluded again in the 4th instar immediately after feeding; but this caused no further increase in the number of plaques as compared with those nymphs which were stretched only in the 3rd instar

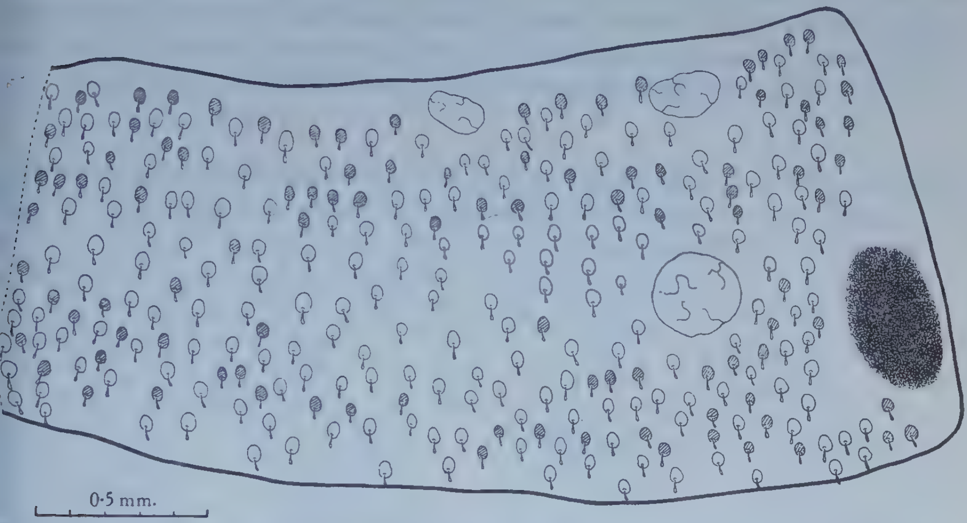
An attempt was made to rear *Rhodnius* from the first instar, occluding the anus at every meal. Most of them died, and only one reached the 4th instar, dying in the attempt to escape from the old skin. This insect gave the following counts on half the third abdominal tergite, the figures in brackets showing the percentage o new plaques at each moult:

1st, 38; 2nd, 79 (108%); 3rd, 153 (94%); 4th, 248 (62%).

The corresponding counts on a normal control were:

1st, 39; 2nd, 84 (115%); 3rd, 128 (52%); 4th, 191 (49%).

As before, the effect of stretching is not apparent until the second moult.



Text-fig. 10. Right half of third tergite in 5th instar nymph, the 3rd instar of which had been excessively stretched by occlusion of the anus. Compare with Text-fig. 8 B.

EFFECT OF PROXIMITY ON THE SIZE OF PLAQUES AND BRISTLES

Although the mutual separation of the plaques does not influence the deter-nination of new plaques until the succeeding instar, it has an immediate effect on he *size* of the plaques and bristles both old and new. There are considerable ifferences in the size of plaques in different regions of the segment; for the purpose f comparison, therefore, the group situated at the centre of the triangle formed by he three muscle insertions has been chosen. Text-fig. 11 shows the plaques in his region in a normal 5th instar nymph (A), in a 5th instar nymph derived from 4th stage in which the anus was occluded after feeding (B), and in a 5th instar ymph derived from an unfed 4th stage (C). It can be seen that both plaques and ristles are enlarged when they are well separated, greatly reduced when they lie lose together.

INFLUENCE OF HORMONES ON THE NUMBER AND SIZE OF BRISTLES AND PLAQUES

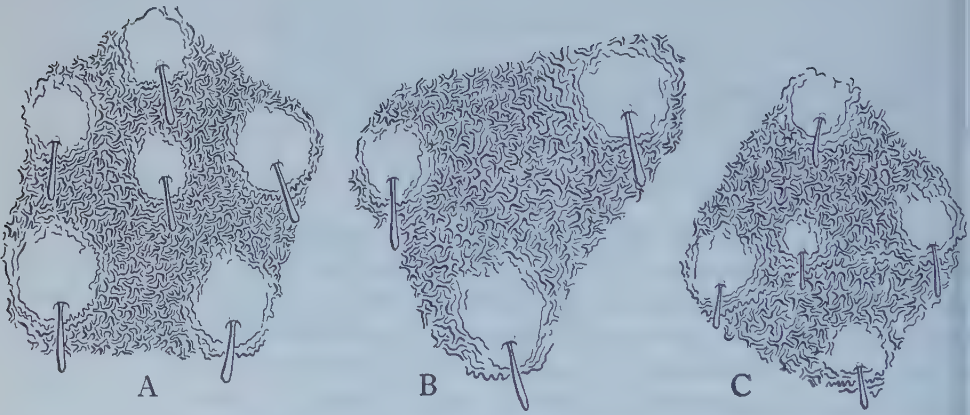
Since the number of new plaques appearing is decided by the mutual separation f the plaques at the preceding moult, must one conclude that at the commence-ent of a given moult the new plaques are already predetermined?

This question has been answered as follows. We have seen that during the moult om the 3rd to the 4th instar, the plaques show an increase of 50·4% (Table II).

Whereas in moulting from the 4th instar to the 5th, the increase is only 27·5 % (Table II). Now in an earlier paper (Wigglesworth, 1936) it was shown that the characters of the various nymphal instars are controlled by the hormone or hormones secreted by the corpus allatum. For example, a 4th instar normally develops 5th instar characters when it moults; but if it is decapitated and exposed to the secretion of the corpus allatum of a 3rd instar it develops 4th instar characters again. If therefore, the new plaques are not predetermined before moulting, a 4th stage nymph caused to moult by decapitating and joining to a 3rd stage nymph with its corpus allatum intact should produce more new plaques than a normal 4th stage nymph.

Table III. *The number of plaques on half the third abdominal tergite in 4th stage nymphs and in the instar derived from these by joining them to 3rd stage nymphs with the corpus allatum present*

	1	2	3	Mean
4th nymph	192	178	191	187
New instar	257	246	262	255
Increase %	33·8	38·2	37·1	36·3

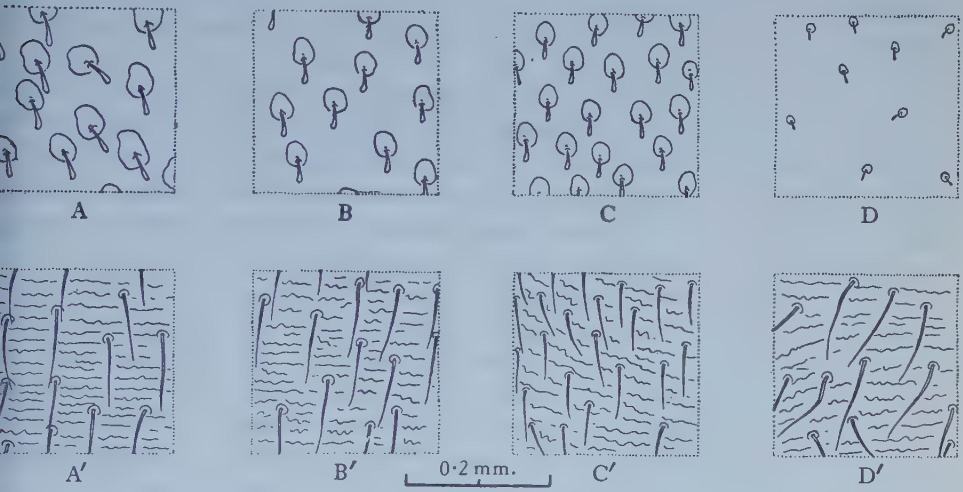


Text-fig. 11. Corresponding groups of plaques in 5th instar nymphs. A, after 4th instar had been fed normally. B, after 4th instar had anus occluded after feeding. C, after 4th instar had been unfed.

Table III gives the results of three such experiments. Instead of the normal increase of 27·5 %, the plaques show an increase of 36·3 %.

It is evident from these results that the number of plaques developed at a given moult is influenced by the hormones to which the epidermis is exposed during moulting. The plaques are clearly not predetermined at the commencement of the moult.

The size of the plaques and bristles is similarly influenced. A group of plaques nternal to the central muscle insertion in a 5th stage nymph ranged in width from 0 to 50μ (average 45μ) with bristles ranging from 36 to 46μ (average 42μ). A orresponding group in an instar produced from a 4th stage nymph by exposure to ne corpus allatum of a 3rd stage, ranged in width from 27 to 40μ (average 37μ) vith bristles of 33 to 40μ (average 37μ). This reduction in size may be a direct ffect of the hormone or it may perhaps be secondary to the increased number of laques; for we have seen that when the plaques are closer together their size is educed (Text-fig. 11).



ext-fig. 12. Approximately corresponding areas of cuticle in the sternites of nymphs above, and dults derived directly from them below. A, A′, normally fed 5th stage nymph. B, B′, normally d 4th stage nymph. C, C′, unfed 4th stage nymph. D, D′, normally fed 1st stage nymph.

FFECT OF PROXIMITY ON THE SIZE OF BRISTLES IN THE ADULT INSECT

In the adult *Rhodnius* bristles are confined chiefly to the sternites and the lateral egion of the tergites. These bristles are derived from the bristles already present the 5th stage nymph, and the effect of mutual proximity on their size has been udied by inducing metamorphosis in nymphs in which the plaques were separated a variable extent.

For example, Text-fig. 12 A, A′, shows the changes in the ventral bristles during ne moulting of a normal 5th stage nymph. Text-fig. 12 B, B′, shows the corre- ponding changes when an imaginal moult is induced in a 4th stage nymph (by ecapitating it and joining it to a moulting 5th stage). The plaques are about equally parated in the 4th and 5th stages and the imaginal spines developed are about the me size in the two cases. But if the same experiment is performed on an unfed h stage nymph, in which the plaques are much closer together, the imaginal ines are very much smaller (Text-fig. 12 C, C′). Finally, Text-fig. 12 D, D′ ows that even when metamorphosis is induced in a 1st stage nymph, the imaginal ines are equal in size to those in the normal adult.

DISCUSSION

The effect of proximity on the size of plaques and bristles is perhaps to be explained quite simply. When the cuticle is stretched the cells which build the plaques are stretched also. They occupy a larger area and therefore lay down a larger plaque. At the same time the cells are more freely exposed to the circulating blood. Perhaps they are better nourished, and perhaps it is merely for this reason that a larger bristle is formed. Conversely, when the plaques are close together, as in the unstretched insect, conditions for the cells are perhaps less favourable and smaller bristles are laid down.

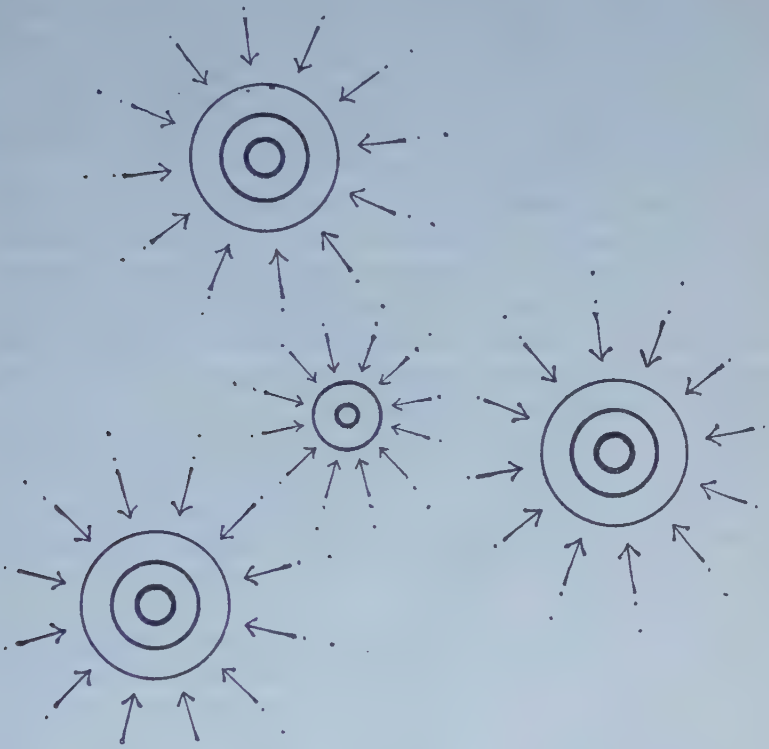
The effect of proximity on the number of plaques is a more important matter. Here the closeness of existing plaques affects the determination of new plaques; and the explanation of this phenomenon may throw light on the whole question of determination. Apart from other factors, dependent on the regional differences in the pattern of the cuticle, each plaque appears to exert an inhibitory influence around it and to prevent the development of new plaques within a certain radius.

It is not, however, the absolute separation of the plaques which determines the radius over which this influence acts. For the number of new plaques that appear during the moulting is the same whether the insect is unfed and the cuticle not stretched at all, or whether it is stretched to the utmost by occlusion of the anus after a full meal. This suggests that it may be the number of cells intervening between plaques which is important; for in the early stages of moulting, this number is of course the same whether the insect has been fed or not.

This idea is supported by the effect of distension at one moult upon the number of new plaques appearing at the next. For when the cuticle has been stretched by blocking the anus, there is a compensatory increase, during moulting, in the number of epidermal cells. For example, in one 4th instar nymph in which the anus had been occluded after feeding in the 3rd stage, a square in the epidermis with a side of 75 μ contained 87 nuclei; and an equal square in a corresponding part of the epidermis of a normal 4th instar nymph contained 84 nuclei. Thus the number of cells per unit area is the same. Consequently, since the plaques are more widely separated, the number of cells which lie between them must be greater in the insect that had been stretched. For example, in these same two insects, the number of nuclei lying within the square formed by four plaques just internal to the central muscle insertion was 148 in the nymph which had been stretched, 119 in the control. If the *effective distance* between plaques is to be measured by the number of cells intervening, we should expect an increase in the number of new plaques appearing when these stretched insects moult again. That is, in fact, what happens (p. 192).

At what stage of development does the determination of plaques occur? The number of new plaques arising at a given moult is influenced by the nymphal stage from which the moulting hormones are provided (p. 194). Hence the new plaques cannot already be determined before moulting begins. Their number is probably nfluenced, as we have seen, by the number of cells intervening between existing laques at the commencement of moulting. Now the number of cells increases apidly after the critical period when moulting is well established. The determinaion of new plaques therefore occurs probably during the early days of the moult r soon after the critical period.

What is the nature of the inhibitory influence exerted by the plaques upon the urrounding cells? Many physical and chemical possibilities come to mind; but s a working hypothesis the following has been adopted (Text-fig. 13). It is supposed



Text-fig. 13. Diagram to illustrate hypothesis of determination of plaques. Explanation in text.

hat as soon as moulting is initiated by the moulting hormones, the existing plaque-orming and bristle-forming centres become active, and draw from the surrounding ells some factor essential for their activity. (As pointed out in an earlier paper Wigglesworth, 1937), protoplasmic bridges connect the epidermal cells with one nother.) The adjacent cells, drained of this essential element, are therefore inapable of realizing their potentialities and forming bristles or plaques. But some ell sufficiently remote from existing plaques is able to become active; it thus forms he centre of a new bristle- and plaque-forming system which likewise draws the essential element" from the zone immediately around it.

There are many phenomena, such as the appearance of new plaques in the unoccupied areas, the inward spreading of plaques across a healed wound, the occasional appearance of contiguous plaques, which can be explained along these lines. But it is scarcely necessary to point out that the mechanism is at present a pure hypothesis, the purpose of which is merely to provide a mechanical analogy for the explanation of the observed phenomena.

It may be pointed out that an inhibitory influence of some kind, preventing the appearance of like organs within a certain limiting radius, has been invoked to explain the appearance of new zooids in colonial organisms such as hydroids. Similarly, a limb disk of a Urodele larva grafted on to the flank of the same animal will develop into an independent limb only if it is sufficiently remote from its original position (Huxley & De Beer, 1934); and Goldschmidt (1921) describes duplication arising in the valves of the external genital organs of certain intersexes of *Lymantria* without injury, which he attributes to an abnormal degree of separation of parts which ought to be in contact.

The nature of the determination process. It is obvious that the epidermal cells must contain the intracellular metabolic systems which are responsible for the types of cuticle they lay down. At an early stage of embryonic development the cells must contain the systems, or the precursors of systems, necessary for the production of all types of cuticle. During development they lose the potency to form particular types and become progressively more specialized. This progressive loss of potency is often called "determination".

But certain cells retain the faculty of laying down several types of cuticle. Thus the general epidermal cells in the *Rhodnius* nymph may lay down ordinary stellate cuticle, or form bristles, sockets or the smooth plaques around them. The process by which one or other of these latent systems is activated, or its mode of action altered in such a way that it leads to the deposition of cuticle of a given type, is again called "determination".

Finally, when the cell is committed to form a bristle, for example, the type of cuticle it will lay down is very different at a nymphal moult and at metamorphosis. The intracellular system is thus subject to a further modification through the action of the hormones controlling metamorphosis. The nature of the action of these hormones will be considered in the next paper, but we may provisionally adopt the hypothesis that by chemical means they modify the action of the intracellular metabolic systems to which we have referred.

To sum up; the following steps are recognized in the development of an animal such as *Rhodnius*. (i) A progressive loss of potency, or a restriction of the range of structures that may be formed by a given group of cells. (ii) An activation of one of the remaining potencies leading to the development of a particular structure. (iii) Metamorphosis, or the modification to a greater or lesser extent of the course of development following upon this activation. Different authors have used the word "determination" to describe the processes by which, at each of these steps, the cells become committed to a particular activity. But in the light of observations to be described in the next paper it seems possible that the change which takes place at the third step is of a different nature from the other two.

Relation of the cells to determination. It was interesting to use the material tudied in order to show the relation of the individual cells to the determination rocess. The experiments on wound healing have shown that when the cells divide epeatedly, and migrate long distances over a wound surface, they still retain their xisting state of determination (p. 183). Their special character is transmitted, pparently indefinitely, to the daughter cells. And this applies not only to those haracters which are manifested at that time (for example, nymphal characters in nymphal insect) but to those characters which will not become apparent until netamorphosis (imaginal characters are equally carried and displaced by the nigration of cells across a healing wound in a nymph) (p. 186). In both cases the ndividual cell and its progeny are all-important in determination. It is therefore ossible to look upon the first process of determination mentioned above (the progressive limitation of potency among certain groups of cells) as a process of orderly 'somatic mutation'' resulting in strains of cells with specific characters, and to egard the organism as a mosaic of such mutant strains.

SUMMARY

The pattern of the dorsal abdominal cuticle in the nymph and adult of *Rhodnius* s described.

If a patch of epidermis is killed by burning, the cells at the margin of the burn livide and migrate towards the centre until the defect is repaired. During this rocess the cells, and the daughter cells derived from them, retain the power of aying down the type of cuticle for which they were originally determined. Conequently a centripetal displacement of the pattern results at the next moult.

Cells migrating in this way in the early nymphal stages carry with them also the rospective imaginal characters. Similar derangements of the imaginal pattern herefore result; but these do not become manifest until metamorphosis.

Thus the individual cells appear to be all-important in the determination of the bdominal pattern.

The nymphal cuticle bears bristles arising from little plaques distributed egularly over the surface. At successive moults new plaques appear in the spaces etween the old ones. If at a given moult the separation of the plaques is artificially ncreased or diminished this has no influence on the number of new plaques eveloped. But if the separation is increased at one moult, the number of new laques may be nearly twice the normal at the succeeding moult.

This result is explained by supposing that each plaque exerts an inhibitory nfluence around it which prevents the development of new plaques; and that the adius over which this influence is exerted is determined not by the absolute istance but by the number of epidermal cells intervening. The nature of the inibitory influence is discussed.

The number of new plaques is not predetermined at a given moult; for it may e increased if moulting is induced by exposure to the hormones of a younger ymphal stage.

If the plaques or bristles are brought close together during the moult, those o the next instar are reduced in size; if widely separated they are enlarged.

At the first moult after a burn the nymphal cuticle at the site of injury is devoi of plaques and bristles. Plaques of normal orientation and distribution are develope at subsequent moults. This regeneration is an exaggeration of the normal process of plaque formation that takes place at each moult.

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EXPLANATION OF PLATE I

Fig. 1. Plaques, bristles, and folds in the epicuticle of a 5th stage nymph.

Fig. 2. Folds in the cuticle and a bristle in the tergites of an adult.

Fig. 3. Epidermis and cuticle of 5th stage nymph mounted whole after staining, to show the distribution of the individual cells between the plaques. The plaques are convex; hence the cells below them are out of focus.

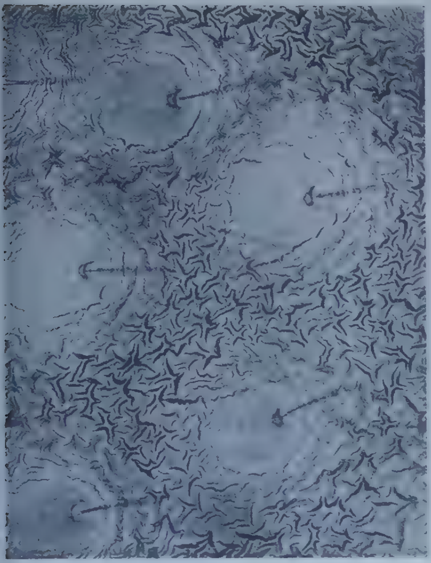

Fig. 1.

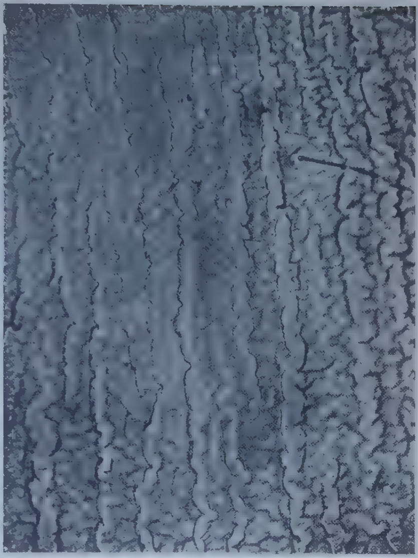

Fig. 2.

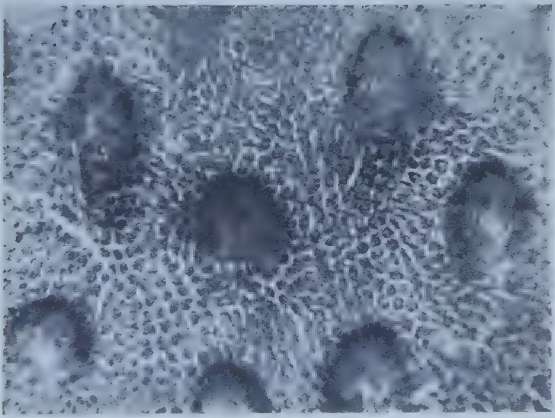

Fig. 3.

WIGGLESWORTH—LOCAL AND GENERAL FACTORS IN THE DEVELOPMENT OF "PATTERN" IN *RHODNIUS PROLIXUS* (HEMIPTERA) (pp. 180–200)

THE DETERMINATION OF CHARACTERS AT METAMORPHOSIS IN *RHODNIUS PROLIXUS* (HEMIPTERA)

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(*Received 22 January* 1940)

(With One Plate and Twelve Text-figures)

Two factors have been recognized as regulating moulting and metamorphosis in *Rhodnius prolixus*: a "moulting hormone" and a so-called "inhibitory hormone" Wigglesworth, 1934, 1936).

(i) *Moulting hormone*. The secretion of this factor is brought about by the tretching of the abdomen by a large meal of blood. Such distension apparently provides a nervous stimulus that is conveyed to the brain. The hormone seems to be secreted in the head, and it was suggested that the corpus allatum is the source f it. But no satisfactory experimental evidence could be produced for this view, which will be refuted in the present paper. The moulting hormone circulates freely n the blood, and after a "critical period" (some 5 days after feeding in the 4th tage, some 7 days in the 5th stage), if the blood is transfused through a capillary ube into other nymphs deprived of the source of hormone (by decapitation while nfed or shortly after feeding), these will be induced to moult. The moulting ormone is present in the blood of each of the five nymphal stages; it initiates cell ivision, which is followed by the deposition of a new cuticle. In the presence of his factor alone, the structural characters of this cuticle, no matter which instar has een caused to moult, are those of the adult insect.

(ii) *Inhibitory hormone*. This factor becomes operative only when growth and moulting have already been initiated by the moulting hormone. It is present in the lood of the first four nymphal stages, and has the effect of preventing the development of the imaginal characters. (It is not inhibitory in the sense of opposing the ction of the moulting hormone, as some authors have assumed). In the presence f this hormone (in addition to the moulting hormone) the insect develops ymphal characters once more when it moults. The inhibitory hormone is secreted y the corpus allatum in the first four nymphal stages. This gland appears to roduce it when exposed to the action of the moulting hormone; nervous stimulation not required. Further, the inhibitory action of the corpus allatum is greater in the arlier nymphal stages than in the later: the gland in the 3rd stage will prevent a moulting 4th stage nymph from developing 5th instar characters and cause it to develop 4th instar characters again.

The purpose of the present work is first to reinvestigate the source of the moulting hormone; and then to consider more closely the mode of action of these two factors in controlling the morphological characters; to find out, in particular, at what stage in development determination of morphological characters takes place and to what extent such determination is reversible.

SOURCE OF THE MOULTING HORMONE[1]

When the study of moulting in *Rhodnius* was first undertaken (Wigglesworth, 1934) many attempts were made to induce moulting in nymphs decapitated 24 hr. after feeding by the implantation of their head contents into the abdomen. These experiments were unsuccessful; and when it was found that moulting could be induced by transfusion of blood from decapitated insects they were abandoned. They have now been taken up again—but with the difference that the organs for implantation have been removed from insects that had just passed the critical period.

In the first series of experiments the brain, together with the suboesophageal ganglion, the sympathetic (oesophageal) ganglion and the corpus allatum, was dissected in Ringer's solution from 4th stage nymphs at 6 or 7 days after feeding or from 5th stage nymphs at about 10 days after feeding. The nerves connecting the sympathetic ganglion with the brain were cut through and the brain plus suboesophageal ganglion then separated from the sympathetic ganglion plus corpus allatum (Text-fig. 1 A). These fragments were then transplanted separately, through oblique cuts at the hind end of the abdomen, into 4th stage nymphs decapitated at 24 hr. after feeding. Nymphs decapitated at this stage never moult unless artificially provided with moulting hormone. Consequently, if moulting could be induced in a single one of them it would afford conclusive evidence that it had been provided with a source of this hormone.

Excluding the few insects (about 10% of the whole) which died, 16 received implants of the corpus allatum and sympathetic ganglion; in two of them corpora allata from two insects were implanted; but although they survived in many cases for more than six months none of them was induced to moult.

On the other hand, of 25 insects which received implants of the brains, 21 moulted. In the absence of the inhibitory hormone of the young corpus allatum they developed imaginal characters.

The brain forms a very much larger fragment than the corpus allatum, and in order to exclude the possibility that in these experiments it served merely to transfer mechanically hormone secreted into the blood elsewhere, large pieces of fat body were transplanted from the donor insects, without washing in Ringer's solution, into decapitated 4th stage nymphs. In five such experiments no moulting was obtained, although the washed brains from the same insects induced moulting.

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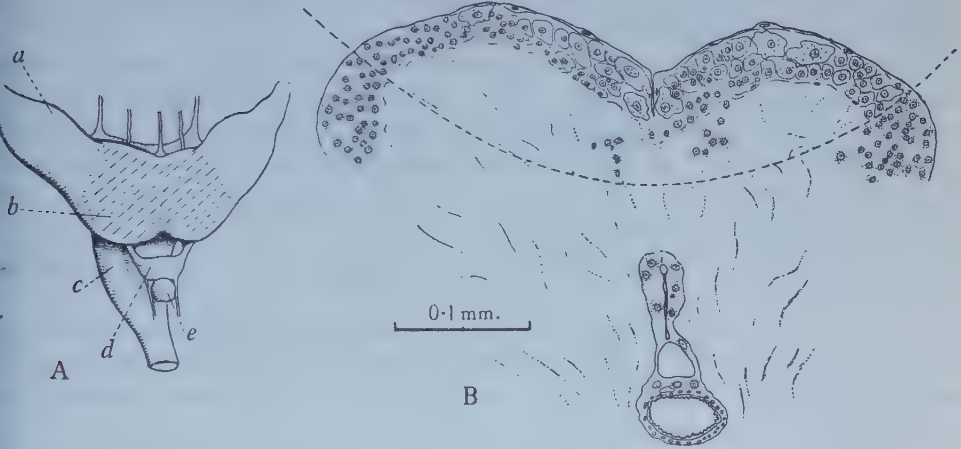
[1] Preliminary note published (Wigglesworth, 1939*b*).

An attempt was then made to locate the source of the hormone within the brain. n preliminary experiments the optic lobes, the dorsal and ventral halves of the entral mass of the brain, and the suboesophageal ganglion were implanted eparately. Positive results were obtained only with the dorsal half of the central nass. The brain was then divided into two parts—a small fragment excised from ne dorsum of the protocerebrum, including the floor of the depression between the rotocerebral lobes, and the remainder of the brain plus the suboesophageal anglion. Out of nine decapitated 4th stage nymphs which received the small orsal fragments, six were caused to moult; out of nine insects which received the est of the brain none moulted. In this case the fragment which gave negative esults was far larger than that which provided the moulting hormone.



ext-fig. 1. A, brain of 5th stage nymph. *a*, optic lobes; *b*, protocerebrum; *c*, suboesophageal nglion; *d*, sympathetic (oesophageal) ganglion; *e*, corpus allatum. The shaded area of the proto-erebrum shows the approximate extent of the excised region. B, vertical section through the posterior rt of the protocerebrum. In the central region above are the large cells with fuchsinophil inclusions; terally are ordinary ganglion cells. The broken line shows approximately the region excised.

In the adult *Rhodnius* Hanström (1938) has described peculiar large nerve cells this dorsal region of the protocerebrum, in which the cytoplasm is often lobulated nd contains rounded masses which stain with acid fuchsin. He suggests that these ay be secreting cells.

In order to see whether these cells show visible changes during the secretion of e moulting hormone, brains of the 4th stage nymphs were fixed in Bouin's olution before feeding and at six days after feeding, sectioned, and stained with hrlich's haematoxylin and van Gieson. Text-fig. 1 B shows a section through the orsum of the protocerebrum. The large cells with the fuchsinophil inclusions are actly as described by Hanström; but no certain differences could be detected etween the cells in the fasting insect and at the height of the secretion of the oulting hormone. Cells of this type were very evident in the disorganized brain ssue when sections were made of the implanted dorsal fragments.

Whether these cells are really the source of the hormone must be left undecided The presence of fuchsinophil inclusions of this kind is commonly regarded a evidence of a secretory function (Scharrer, 1937); but it may be pointed out tha the cells of the corpus allatum, which are almost certainly the source of the inhibitory hormone, do not stain in this way; they are either vacuolated or have dense and homogeneous or very finely granular cytoplasm.

THE EARLY HISTOLOGICAL CHANGES PRODUCED BY THE "INHIBITORY HORMONE"

The histological changes in the epidermis and associated tissues of the abdomen during moulting have already been described in detail (Wigglesworth, 1933). Bu in order to throw light on the mode of action of the "inhibitory hormone" these changes have been reinvestigated.

The entire dorsum of the abdomen has been fixed with Carnoy's fixative stained with haematoxylin, and mounted whole on successive days after feeding three preparations being usually made for each day. Omitting the changes tha occur in the oenocytes and dermal glands, which have already been described, the changes in the epidermis, at 24° C., when the 5th stage nymph moults to an adult may be summarized as follows.

1st day after feeding: no visible change. Chromatin still scattered throughout the nuclei.

2nd day: "activation" of the cells begins; i.e. the cytoplasm becomes increasingly dense and the chromatin becomes clumped at the centre of the nuclei (cf. Wigglesworth, 1937). This change occurs all over the abdomen but is most marked at the intersegmental membranes and at the margins.

5th day: mitosis begins at the margins of all the segments and over the general surface of the first segment and the two terminal segments (Text-fig. 2 A) Chromatin droplets derived from dead nuclei begin to appear.[1]

6th day: mitoses begin at the central point of the intersegmental membranes They are now exceedingly numerous in the lateral, anterior and posterior regions of the abdomen—that is, in the regions where morphological change is greates (Text-fig. 2 B).

7th day: mitoses very numerous at the central point of the intersegmenta membranes (Text-fig. 2 C).

8th day: vacuolation appears in the apical parts of the cells all over the tergites

9th day: mitoses now occur all along the intersegmental membranes (Text-fig. 2 D).

10th–11th day: cells appear to be detached from the cuticle all over the tergites A very *few* mitoses occur on the general surface remote from the margins and th intersegmental membranes. Chromatin droplets begin to appear below the bristle and plaques.

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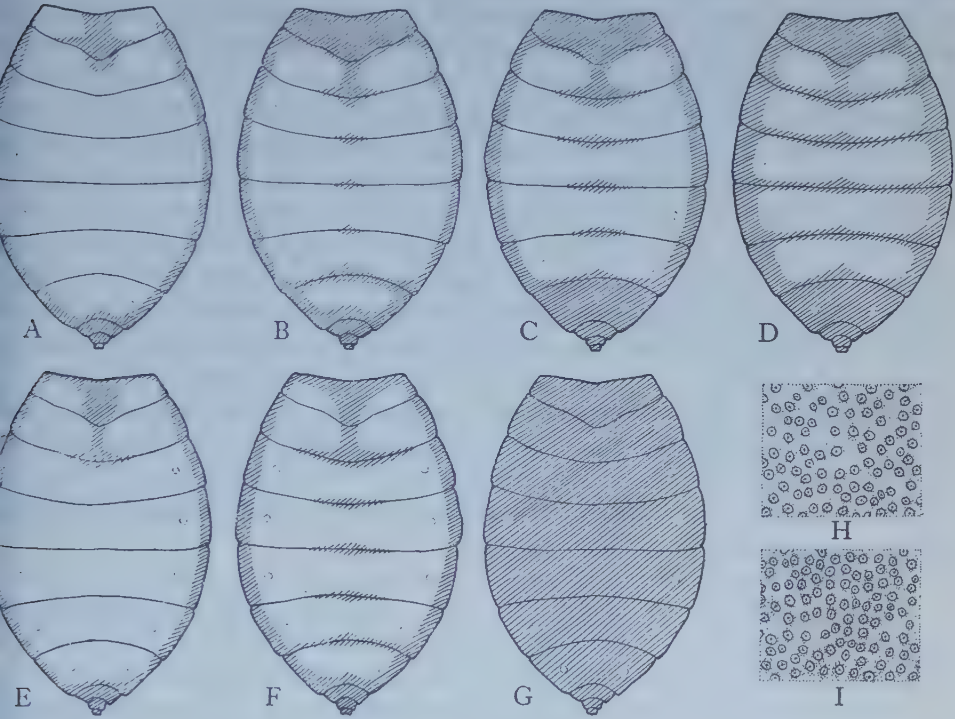
[1] The significance of these chromatin droplets and the evidence that they are derived from defunc cells will form the subject of a separate paper.

13th day: formation of chromatin droplets occurs over the general surface of the egments, particularly around the plaques.

16th day: a few mitoses still occurring. Formation of chromatin droplets still progress.

18th day: deposition of new cuticle begins.

28th day: moulting occurs.



ext-fig. 2. Shaded areas show the approximate distribution of mitoses during the moulting of 5th ge nymph; A–D, imaginal moult. A, 5 days after feeding; B, 7 days; C, 8 days; D, 9 days. E–F mphal moult. E, 5 days; F, 6 days; G, 7 days. H–I, density of cells in approximately correonding areas of tergites after the completion of mitoses; H, imaginal moult; I, nymphal moult.

The first change visible in the cells is their general "activation". This occurs the second day after feeding and affords evidence that the moulting hormone is ready being secreted at this stage. The "critical period", which occurs at about e seventh day, corresponds with the stage at which mitoses are becoming general. hey have already begun in the peripheral regions of the abdomen by the fifth day. those insects which, under experimental conditions, occasionally show partial oulting, this is confined to those regions in which mitosis occurs earliest. It is orth noting that there is no sign of a "wave" of mitoses passing from before ckwards over the abdomen, such as has been described by Kühn & Piepho (1938) Lepidoptera larvae.

If the 5th stage nymphs are decapitated and caused to moult by joining them to other 5th stage nymphs that have passed the critical period, the same change take place in the abdomen. But they appear more rapidly—doubtless because th moulting hormone is supplied earlier in higher concentration. Within four days th normal seventh day stage is reached; by the tenth day mitoses have almost ceasec and the normal fifteenth day stage is reached.

During the moulting of the 4th stage nymph to produce the 5th stage the histo logical changes are at first similar to those just described; but mitoses later becom numerous throughout the epidermis and other differences are to be observed. In order to prove that these differences are due to the presence of the "inhibitory hormone" a number of 5th stage nymphs were decapitated 24 hours after feeding and caused to moult by joining them to 4th stage nymphs with the corpus allatum intact. The histological changes in the 5th stage nymphs, which, owing to the presence of the inhibitory hormone, now undergo another nymphal moult, were studied as before.

Cross-circulation in this way causes, as we have seen, a considerable acceleration of the moulting process. In order to allow a better comparison with the changes already described in the normal moulting of the 5th stage nymph, the times in the summary which follows have been corrected as far as possible so as to eliminate this accelerating effect.

5th day after feeding: "activation" everywhere; mitoses numerous in the marginal zone and at the anterior and posterior extremities of the abdomen (Text-fig. 2 E).

6th day: mitoses very numerous laterally and just beginning at the middle of the intersegmental membranes (Text-fig. 2 F).

7th day: mitoses numerous along the intersegmental membranes.

8th day: mitoses exceedingly numerous not only along the intersegmenta membranes but all over the general surface of the segments. They are mos abundant above the heart, in the mid-line of the tergites (Text-fig. 2 G).

10th day: mitosis almost complete; cells densely packed all over the surface and readily detached from the cuticle. Chromatin droplets appear in small numbers here and there.

12th day: deposition of new cuticle begins.

These times are subject to much variation, but the chief differences between nymphal moult and an imaginal moult are clear. At first the distribution of mitose is similar; but soon after the critical period they occur in great numbers all over th abdominal segments during the nymphal moult, whereas during the imaginal moul they are practically confined to the intersegmental membranes and the peripher of the abdomen. Hence, when the period of cell division is complete, the numbe of cells per unit area is much greater at a nymphal moult (Text-fig. 2 H, I). Th formation of chromatin droplets, indicating the death of existing cells, is much mor evident at the imaginal moult.

In the converse experiment, when an imaginal moult is induced in a decapitate 4th stage nymph by joining to a 5th stage nymph, the histological changes are thos of the imaginal moult.

SWITCH OVER FROM NYMPHAL TO IMAGINAL DEVELOPMENT

It is clear from the foregoing account that the histological changes in the pidermis of the abdomen—the universal “activation” of the cells, followed by itoses in the marginal zone and later along the intersegmental membranes—are e same whether a nymphal or an imaginal instar is to be produced. Indeed, in the arlier papers on moulting in *Rhodnius* evidence has already been produced that at ll instars growth is initiated by the same moulting hormone, and that the “in-ibitory hormone”, responsible for the production of nymphal characters, is secreted, r is able to exert its action, only when the process of moulting has already been arted. It was shown, for example, that by decapitating the early nymphal stages round the critical period, and thus eliminating the source of the inhibitory ormone, it is possible to obtain a graded series of forms ranging from normal ymphs, through all degrees of intermediates, to precocious adults (Wigglesworth, 934).

These intermediate forms were believed to represent insects which had begun eveloping nymphal characters and then, as a result of a deficiency of “inhibitory ormone”, had continued with an intermediate type of development.

In these experiments there is not, of course, a complete removal of the inhibitory ctor at a given stage. Any quantity of this factor present in the blood at the time f decapitation either persists in the blood, or is absorbed by the tissues, and there-re is able presumably to continue to exercise its effects throughout development. here is not a complete “switch over” in development.

SWITCH OVER FROM IMAGINAL TO NYMPHAL DEVELOPMENT

It was of interest to perform the converse experiment: to allow nymphs to start eveloping towards adults and then at different stages in the process to expose the ssues to the inhibitory hormone and to see to what extent they could be induced switch over to a nymphal type of development.

The experiment was carried out as follows. A large batch of 5th instar nymphs as fed, and at daily intervals thereafter several of them were decapitated and joined 4th instar nymphs, six or seven days after feeding, in which the anterior part of e head had been removed but the corpus allatum retained. At 24° C. the interval etween feeding and moulting in the 5th stage nymph averages 28 days. The periment was carried out at daily intervals from the time of feeding until the venty-seventh day. As illustrating the simplicity of the technique it may be corded that out of 160 insects joined for this purpose only one pair died before oulting took place.

It is evident that in these experiments the 5th instar nymphs developed as agines up to the time of decapitation. Thenceforward they received inhibitory rmone. They certainly received small quantities of this hormone within the next our or two; but it would probably be several days before the corpus allatum of the

4th stage nymphs could raise the concentration of this hormone up to the norma level in the blood of both insects. The "switch over" in development must therefor be gradual. It is also subject to considerable variation; due in part to the insects o a given day having reached slightly different stages in the moulting process, bu probably due chiefly to differences in the efficiency of mixing of the blood from th two insects after joining. The time-table which follows is therefore to some exten arbitrary—it is based on the most successful effects produced at a given day. Th only characters studied are those visible in the dorsal surface of the abdomen.

1st–5th day: (Pl. I, fig. 1). If the switch over occurs before the critical perio the characters of the dorsum of the abdomen are entirely nymphal. Many new plaques and bristles have made their appearance, particularly in the lateral parts o the segments (Text-fig. 3 A).

7th day: (Pl. I, fig. 2). The pigment pattern of the abdomen is that of a norma nymph; except at the hinder end of the seventh segment where the pigmentatio and the type of cuticle are imaginal. Almost all the plaques in the general cuticle are normal in form, but a few have disappeared or are imperfect (Text-fig. 3 B).

8th–9th day: (Pl. I, fig. 3). The general pigmentation is nymphal but the side of the segments show traces of imaginal pigmentation at their anterior margins. The first segment and the terminal segments are becoming imaginal in form. There is n sign of the hinge line (Wigglesworth, 1940) except on the seventh segment. The cuticle of the general surface has the stellate folding characteristic of the nymph, bu many of the plaques have disappeared, particularly in the central region of eac segment. When plaques are present many of them have defective bristles or non at all (Text-fig. 3 C).

10th day: the imaginal area on the seventh segment is spreading further forward The general cuticle now has very few plaques, but numerous pale areas in which the epicuticle is unpigmented are visible where they previously existed (Text-fig. 3 D)

11th day: (Pl. I, fig. 4). The nymphal pigment spots are present and at the sam time a mottled pigmentation extends over the area normally occupied by th imaginal pigment spots.

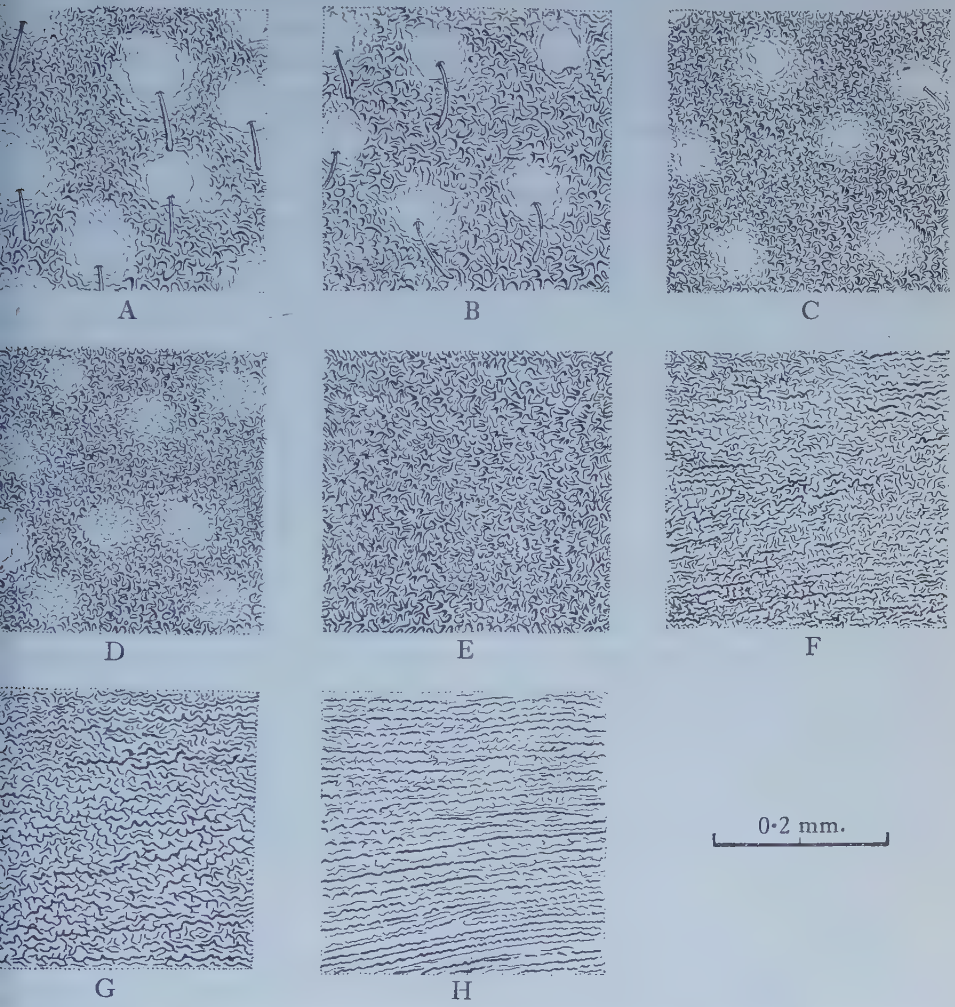
12th day: (Pl. I, fig. 5). The imaginal area extends almost all over the seventh segment. The hinge line is well developed in this segment but not elsewhere. The abdominal plaques are still more scarce.

13th day: (Pl. I, fig. 6). The hinge line now extends forward to the sixth segment and traces of it are visible on most of the remaining segments. The pigment spot combine both adult and nymphal distribution. The general cuticle is of nympha type, but plaques are practically absent (Text-fig. 3 E).

14th day: (Pl. I, fig. 7). Imaginal cuticle present in the intersegmental region and at the sides of the abdomen. The hinge line is present throughout, though no fully formed. Imaginal pigment spots are present and normal; nymphal pigmen spots are becoming incomplete. The cuticle is of nymphal type in the central par of the segment, but plaques are absent; it becomes imaginal towards the margin (Text-fig. 3 F).

16th day: apart from a small area of intermediate cuticle in the central part o ch segment (Text-fig. 3 G), pigmentation and cuticle structure are entirely aginal.

17th day onwards: (Pl. I, fig. 8). Pigment pattern and cuticle structure are of rmal imaginal type (Text-fig. 3 H).



ext-fig. 3. Typical areas of the cuticle in tergites of insects produced from 5th stage nymphs switched er from imaginal to nymphal development at different times after feeding. A, 5 days; B, 7 days; 9 days; D, 10 days; E, 13 days; F, 15 days; G, 16 days; H, 17 days. All drawn over photographs d then bleached.

These experiments prove that the determination of the epidermis to form imaginal ticle proceeds at a different rate in different parts of the abdomen. Many parts main capable of reverting to a nymphal type of development, under the influence the inhibitory hormone, long after the critical period; while other parts are early mmitted irrevocably to lay down imaginal structures.

When this sequence of changes is compared with the normal succession histological changes in the epidermis, as described above (p. 204), the two are see to be closely related. Activation and cell division, with the formation of chromat droplets as the result of cell death take place earliest in the first and the termin segments and along the margins of the abdomen. And these are the regions whic first become irrevocably determined. Chromatin droplets begin to appear benea the plaques and bristles about the tenth day. It is on the eighth or ninth day that switch over to nymphal development results in plaques with bristles wanting imperfect; while after a switch over on the tenth or eleventh day the plaques a almost absent.

The appearance of chromatin droplets is often due to the death of cells whic had some specialized function in the nymph—for example, bristle-forming cell When these cells die this function is necessarily lost. But it is certain that in man cases the same cell may have its function altered by the inhibitory hormone. striking example is seen where the switch over occurs about the tenth day. Man of the bristle-forming cells have already succumbed, so that bristles are absent. I many places the cells which surrounded them are incapable of laying down th characteristic cuticle of the plaque. They can, however, lay down ordinary nymph epicuticle with stellate folds and they retain the faculty of secreting an epicutic that is unpigmented, as it normally is over the plaques (Text-fig. 3 D). From th it is evident that the various faculties of a given cell can be determined to som extent independently of one another.

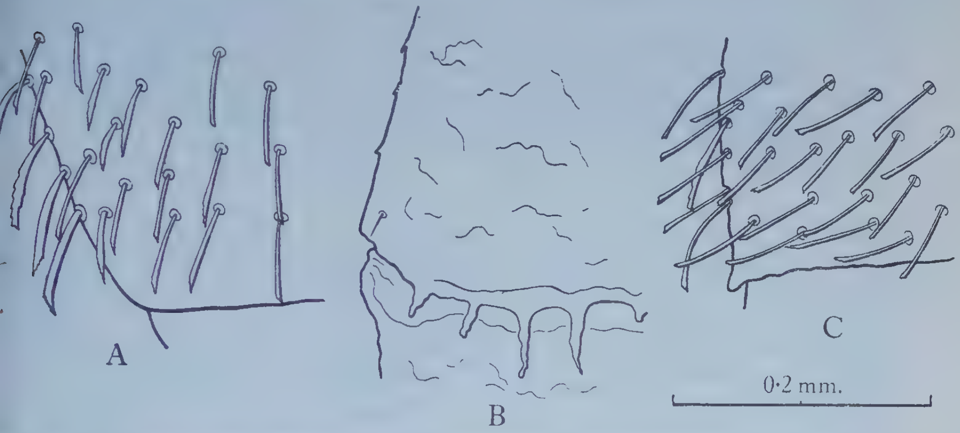
Finally, it is interesting to note that the type of cuticle to be laid down by th cells over the central parts of the abdominal segments is still capable of reversal tw or three days before its deposition is due to begin. Deposition of cuticle commenc about the eighteenth day; a switch over on the fifteenth day results in nymph cuticle on parts of the segments; a switch over on the sixteenth day results in sma areas of intermediate cuticle. But once the deposition of cuticle has begun, neith its form nor its pigmentation (the pigmentation does not appear until after moultin about the twenty-eighth day) can be influenced by the inhibitory hormone.

INDUCTION OF MOULTING IN THE ADULT

The question now arises as to whether the epidermis of the adult retains th faculty of laying down a new cuticle; and whether the characters of this cuticle ca be influenced by the hormones present.

When studying the effect of the secretion from the corpus allatum on eg production in the adult female, 5th stage nymphs of *Rhodnius* were joined, soo after the critical period, to decapitated adults; but in no case was the adult cause to moult. From these experiments it was concluded that the adult is incapable renewed ecdysis (Wigglesworth, 1936). But later it was found that the adult inse is able to repair wounds, with the local formation of new cuticle, and that th histological changes in the epidermis during this process are identical with thos occurring during moulting (Wigglesworth, 1937). Meanwhile Furukawa (1935) ha nown that if the antenna of an adult earwig (*Anisolabis*) is transplanted on to the ase of the cercus of a nymph of the same species it will moult again simultaneously ith the nymph; and recently Mauser (1938) has shown that the imaginal fore-limb f *Dixippus* implanted on to the stump of a limb of a 5th or 6th stage nymph can be duced to moult again and to develop traces of the red colouring on the inner aspect f the femur, which is an imaginal character.

It seemed probable therefore, that the failure of the adult *Rhodnius* to moult in e earlier experiments was due to an insufficient quantity of moulting hormone. cting on this notion the small adult of the bed-bug *Cimex* was joined after decapiation to the relatively large 4th or 5th stage nymph of *Rhodnius*. As stated in a reliminary note (Wigglesworth, 1937), this experiment was successful, and the *imex* adults were duly caused to moult.



ext-fig. 4. Corresponding areas at posterior angle of third abdominal tergite in *Cimex*. A, normal lult; B, instar produced by inducing moulting in an adult 3 months old. C, instar produced by ducing moulting in an adult less than 24 hr. old.

The new cuticle laid down by the adult *Cimex* in these experiments was more or ss formless. The intersegmental membranes of the abdomen were recognizable, ut the cuticle was practically devoid of hairs or spines and had developed along e margins of the segments a number of curious non-articulated outgrowths of zarre shapes (Text-fig. 4 B). The epidermis of the adult *Cimex* thus appears to ave lost the faculty of laying down a cuticle of characteristic form.[1] But the adults sed in these experiments were at least 3 months old and it seemed possible that in ounger adults this faculty might be retained. When the experiment was repeated ith adults 3 weeks old, the same result was obtained; but on joining adults within hr. of moulting, at a time that is when the epidermis is still engaged in secreting e inner layers of the new cuticle, they were caused to moult again and to produce cuticle covered with well-formed bristles (Text-fig. 4 C). *Cimex* adults 3 days old acted similarly; the precise stage at which the faculty to form bristles is lost has ot been determined.

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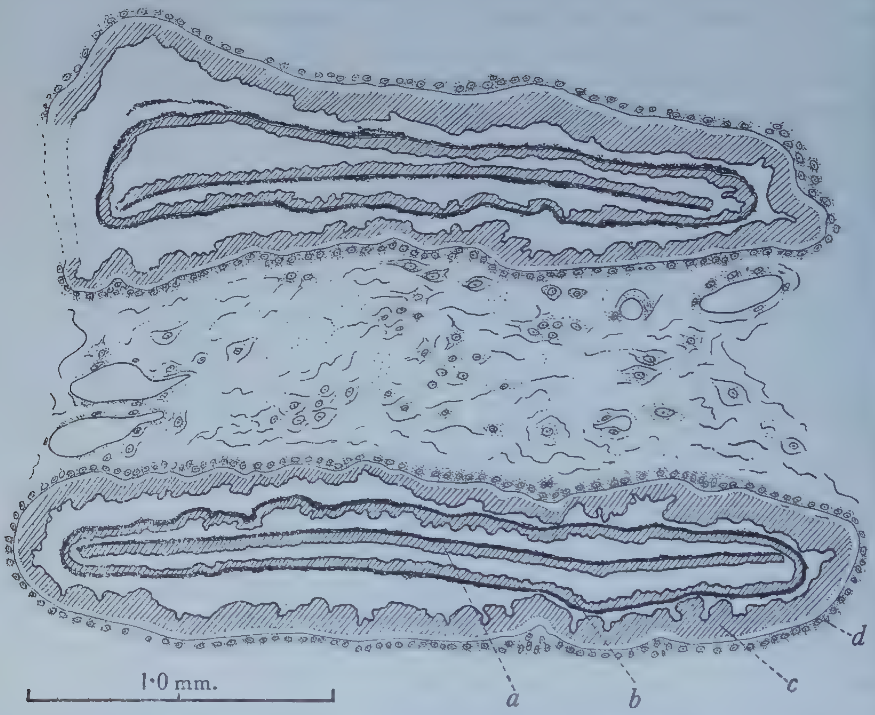
[1] Piepho (1938*b*), has shown that a fragment of imaginal cuticle of the moth *Galleria* implanted o young pupae lays down a similar formless cuticle without scales.

It was shown in an earlier paper that if cylindrical segments of the head o
Rhodnius nymphs are implanted in the abdomen of nymphs which are moulting
the epidermis grows outwards to restore continuity with itself and then the en
capsulated cuticle proceeds to moult (Wigglesworth, 1936). This method has bee
employed to prove that the epidermis of the adult *Rhodnius* is capable of moultin
again. Fragments of the femur or tibia, ½ to 1 mm. in length, were implanted int
the abdomen of 4th stage nymphs 24 hr. after feeding. In one such experiment th



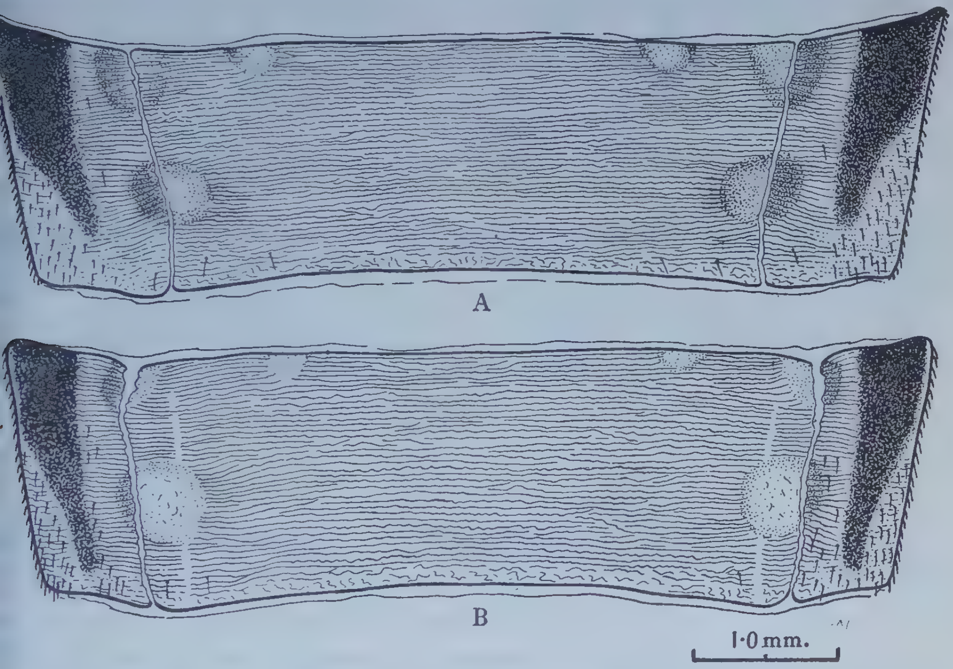
Text-fig. 5. Longitudinal section of fragment of tibia of adult *Rhodnius* implanted in 4th stag
nymph and fixed when this host became adult. *a*, original cuticle; *b*, first new cuticle forming con
tinuous capsule; *c*, second new cuticle forming continuous capsule; *d*, epidermis. The exocuticle i
shaded in each case. The partially digested endocuticle beneath *a* and *b* stains deeply with haemo
toxylin.

implantation was made on 19. v. 1936; the 4th stage nymph moulted to the 5t
instar on 9. vi. 1936; it moulted again to become adult on 15. vii. 1936, and th
implant was removed and sectioned a few days later. The result is shown in Text
fig. 5, from which it can be seen that the fragment of adult tibia has been caused t
moult twice more. (This method of implanting fragments of cuticle has recentl
been used extensively by Piepho (1938 *a*, *b*: 1939 *a*, *b*) for studying the control o
moulting and metamorphosis in Lepidoptera (see p. 216).)

Since it was evident from these experiments that the epidermis of the adul
Rhodnius will respond to the moulting hormone, a renewed attempt was made t
induce moulting in the whole insect. Adults were decapitated at a time when ther

as plenty of blood in the stomach; a 5th stage nymph about 10 days after feeding, ith the anterior part of its head removed, was joined to the neck of the adult; and second 5th stage nymph was joined to the abdomen—a small window about 1 mm. juare being cut in one of the sternites and the abbreviated head of the nymph serted into this. A single adult now received blood from two 5th stage nymphs, nd it was caused to moult simultaneously with these.

Some ten adults were caused to moult in this way. At the time of joining they aried in age from a few days to several months; but, unlike the *Cimex* adults, no

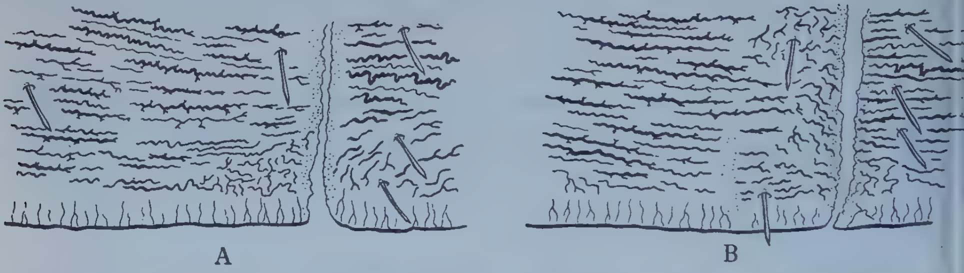


xt-fig. 6. A, tergite of fifth abdominal segment of adult *Rhodnius*. B, new cuticle of the same after moulting induced with the blood from 5th stage nymphs.

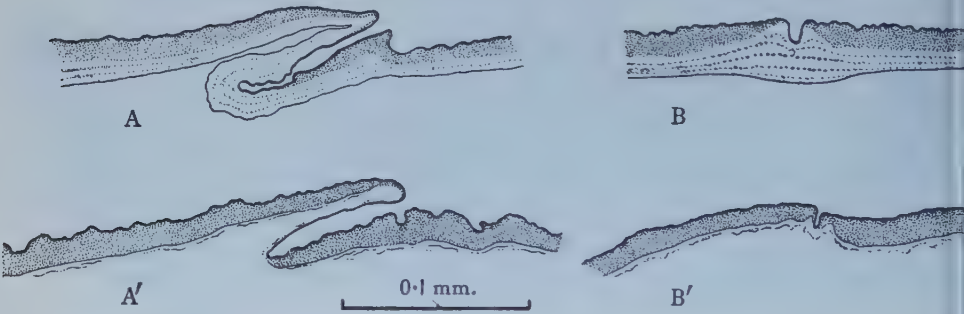
atter what their age, they laid down a new cuticle of normal form and pigmentaon, furnished with bristles of the usual type—though the cuticle may occasionally ow small imperfections, and some of the bristles may be ill-formed. The characters this cuticle are purely imaginal. This is seen in the distribution of pigment in the rgites, in the presence of a "hinge line", in the form of the bristles and the type folding in the epicuticle (Text-figs. 6, 7).

During moulting the oenocytes become enormously enlarged, as they do in oulting nymphs, and the dermal glands become active. In the new cuticle the ouths of the dermal glands often extend to form finger-like outgrowths—at first staken for vestigial bristles.

It is worth noting that during moulting in the adult the endocuticle is dissolved, it is in moulting nymphs (Wigglesworth, 1933). Consequently, at points where an exocuticle is wanting, such as the hinge line and the intersegmental membrane the old cuticle becomes excessively fragile and breaks at a touch (Text-fig. 8). It also worth noting that in females which were caused to moult, the development o eggs was arrested during the process. This is further evidence that the hormone necessary for moulting differ from those needed for egg production (Wigglesworth 1936).



Text-fig. 7. A, posterior margin of fifth abdominal tergite of adult *Rhodnius* showing part of th hinge line. B, the same region of the cuticle after moulting induced with the blood from 5th stag nymphs.



Text-fig. 8. A, longitudinal section of intersegmental membrane in normal adult *Rhodnius*. A′, th same after moulting, showing digestion of endocuticle. B, transverse section of hinge line in norm adult. B′, the same after moulting.

REVERSION TO NYMPHAL CHARACTERS DURING MOULTING IN THE ADULT[1]

The next question is whether the epidermis of the adult insect, exposed to th "inhibitory hormone" during moulting, can lay down again a cuticle with nympha characters. This was first tested in 1936 by excising a piece of cuticle about 1 mm square from one of the tergites of a 4th stage nymph soon after feeding, implantin in its place a similar fragment removed from an adult, and sealing the edges wit paraffin of low melting point. When the 4th stage nymph moulted, the cuticle lai down at the site of the implant, though devoid of plaques, had the epicuticle throw into star-shaped folds exactly like those of the nymphal cuticle elsewhere. I appeared, therefore, that under the influence of the inhibitory hormone of the 4t ge nymph the adult epidermis had formed a nymphal cuticle. There remained, wever, the possibility that in these experiments the imaginal epidermis had died, d the new cuticle was secreted by nymphal cells that had spread in from the argins of the wound.

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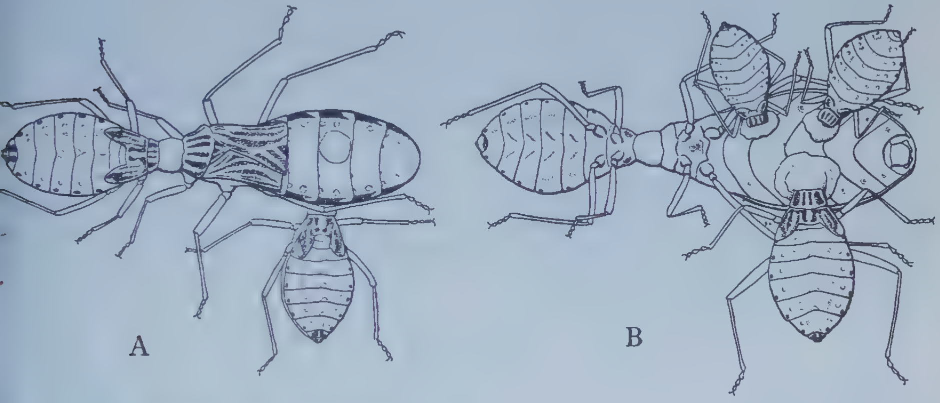
[1] A preliminary note on these results has already been published (Wigglesworth, 1939*a*).

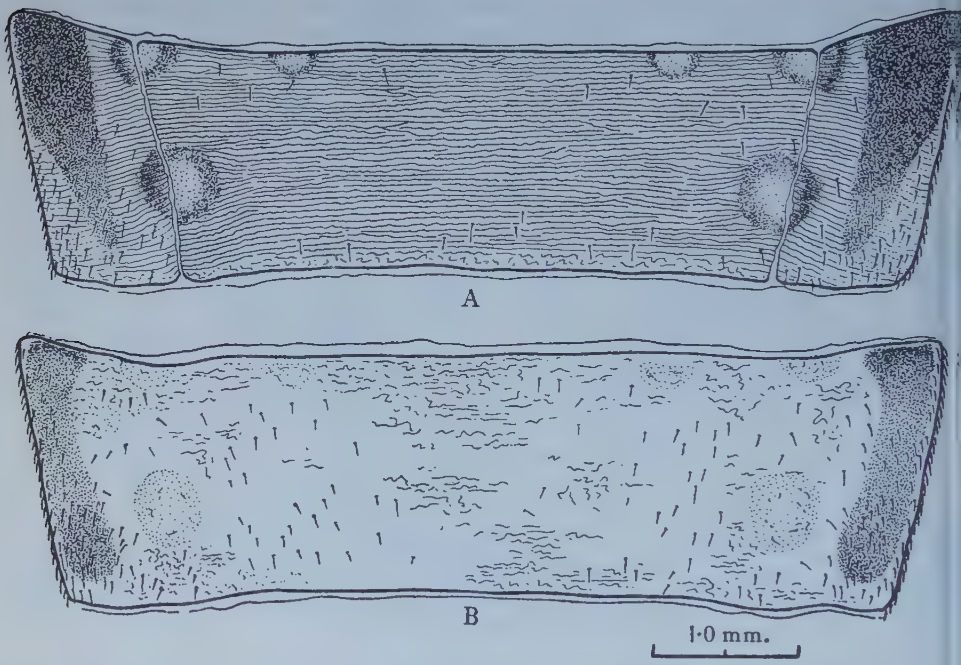
An attempt was therefore made to induce moulting in the whole insect by ning it to 4th stage nymphs with the corpus allatum intact. For some unknown ason these experiments failed. When moulting was not induced by two 4th stage mphs, as many as five (one on the neck and four on the abdomen) were joined a single adult, but without success. Whether this failure was due merely to chance r even after joining to 5th stage nymphs, the adults occasionally fail to moult) whether the imaginal tissues respond less readily to the moulting hormone from e 4th stage is not known.



xt-fig. 9. A, adult *Rhodnius*, with wings cut short and corpora allata of 4th stage nymphs im- nted in abdomen, decapitated and joined to two 5th stage nymphs. B, adult *Rhodnius*, decapitated l joined to two 5th stage nymphs and two 4th stage nymphs.

The experiment was finally carried out as follows (Text-fig. 9). The adult was used to moult as before by joining one 5th stage nymph to the head and a second the abdomen; and in addition it was provided with the secretion from the corpus atum of the 4th or some earlier stage. This was done in several ways in an effort produce the maximum effect. (i) One or two 4th or 3rd stage nymphs with the rpus allatum present were joined to the abdomen in the same way and at the same ne as the 5th stage nymphs. (ii) The hind part of the head, containing the corpus atum, was removed from five or six nymphs in the 1st, 2nd, 3rd or 4th stages and planted through an incision in the abdomen of the adult. (iii) Brains plus corpora ata or corpora allata alone were dissected out of six 4th stage nymphs and im- nted in the same way. In each case the 5th stage nymphs served as indicators the presence of the inhibitory hormone in the circulating blood; for when this s present they developed nymphal characters again.

Altogether 24 adults were caused to moult while exposed to the inhibitory rmone by these means. When the characters developed by the 5th stage nymphs indicated that appreciable quantities of inhibitory hormone were present, the ne cuticle of the adult always showed signs of a partial reversion to nymphal character This change did not affect elaborate structures such as the genitalia. But in th most successful experiments it was evident in the general pattern of the abdome (Text-fig. 10). The distribution of pigment at the sides of the segments showed partial return to the nymphal arrangement; the hinge line was absent; the cutic of the lateral pleat (which is highly elastic and devoid of an exocuticle in the norm adult) developed in places a pigmented exocuticle; a considerable number of ne



Text-fig. 10. A, fifth abdominal tergite of adult *Rhodnius*. B, the same segment after moultin induced by two 5th stage nymphs and the implantation of brains and corpora allata from six 4 stage nymphs. The bristles lying between the hinge lines in A, and in the corresponding area in are copied exactly from the specimen. Compare Text-fig. 6.

spines appeared in places where no spines existed before;[1] the form of these spin was intermediate between those of adult and nymph, and they were often surrounde by a small plaque of nymphal type; and finally the new epicuticle showed th characteristic star-shaped folding of the nymph (Text-fig. 11 B).

In experiments carried out at the same time as these, Piepho (1939 *a*, *b*) showe that when fragments of pupal cuticle from the wax-moth *Galleria* were caused t moult by implantation into young larvae, they laid down again a cuticle of larv type. This effect was most readily induced in that region of the epidermis whic had grown outwards to restore continuity with itself ("Umwachsungshypodermis" the case of *Rhodnius* it was observed in several cases that the nymphal form of e cuticle was most marked at the site of the implantation of the corpora allata. nis result may perhaps be comparable with that obtained by Piepho—a conseence, that is, of the wound-healing process. Or it may be due simply to this gion of the cuticle being closer to the implanted glands. When small burns were ade in the cuticle remote from the site of the implantation they did not seem to oduce this effect.

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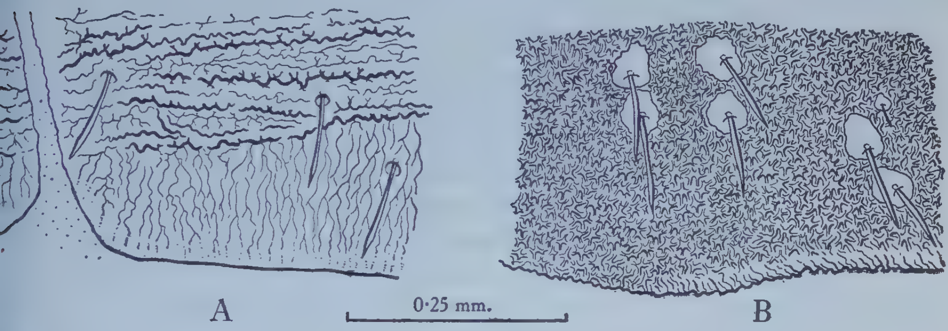
[1] The adult represented in Text-fig. 10 had more spines than usual on the segment illustrate and more new spines appeared at moulting than in any other experiment in this series.

Piepho (1939*a*) also observed that the epidermis immediately under the imanted fragment of pupal cuticle ("Stammbereich") often laid down cuticle of ipal type on moulting for the first time in a young larva, but produced larval ticle if caused to moult a second or a third time. In the hope of obtaining a more mplete reversion to nymphal characters in *Rhodnius* many attempts were therere made to induce adults to moult a second time by removing the first set of



xt-fig. 11. A, posterior margin of fifth abdominal tergite of adult *Rhodnius*, including part of ige line. B, the same area after moulting induced by two 5th stage nymphs and the implantation brains and corpora allata from six 4th stage nymphs. Compare Text-fig. 7.

ymphs when they had moulted and applying a new set. Decapitated adults which d moulted once (they were still enclosed, of course, in their original cuticle as ell as the new one) remained alive for more than 3 weeks after the first moult; but ly in one insect was a second moult induced. Unfortunately, for some reason, e cuticle and spines of both new skins had very ill-defined characters in this sect.

When the dorsum of the abdomen in these adult insects was removed and ined during the course of moulting, it was found that if moulting was induced the moulting hormone from 5th stage nymphs alone, mitoses were confined to e periphery of the abdomen and to the intersegmental membranes; whereas if posed also to the "inhibitory hormone", mitoses were numerous over the entire ea of the segments. As a result, the epidermal cells per unit area were far more merous in those which resumed their nymphal characters than in those in which e adult characters were retained (cf. p. 204 and Text-fig. 2 H, I).

DISCUSSION

Source of the moulting hormone

It was suggested in an earlier paper (Wigglesworth, 1934) that the moultin hormone might be secreted by the corpus allatum. This suggestion was based o the histological examination of the head contents during the early stages of moultin in the course of which it was found that during the critical period the cells of th corpus allatum became swollen and their cytoplasm dense. This evidence was no very convincing, for the histological changes in the epidermal cells show that th moulting hormone is already being secreted within 48 hr. of feeding, several day before the critical period. Unfortunately, the corpus allatum of *Rhodnius* is s placed that it has not proved possible to remove it without damaging the brain o its blood supply. No satisfactory experimental proof of this hypothesis was there fore produced. Attempts at section of the nerves from the brain to the corpu allatum gave inconclusive results.

In a later paper (Wigglesworth, 1936) an attempt was made to prove this hypothesis by showing that nymphs with the brain removed, but with the corpus allatu intact, provide a better source of moulting hormone than those decapitated behin the corpus allatum. But in view of the results described in the present paper, it probable that the apparent success of these experiments was due to the inclusion o a small piece of the posterior dorsal region of the brain with the corpus allatum.

In the present work we now have good evidence that the moulting hormone secreted by the brain; probably by cells located in the dorsum of the protocerebrum It may be well to compare this conclusion with those reached in other groups o insects.

In Lepidoptera, removal of the corpora allata does not prevent pupatio (Bounhiol, 1938). It was originally suggested by Kopeć (1922) that in this grou a hormone causing pupation is secreted by the brain; and Bounhiol (1938) has con firmed that removal of the brain before the critical period in the silkworm and othe caterpillars arrests pupation. He is, however, inclined to attribute this, not to th production by the brain of a hormone, but to its nervous control of the function o the gut. (Bounhiol indeed does not consider that moulting or pupation hormone have been satisfactorily demonstrated in any group of insects). Attempts to induc pupation in caterpillars deprived of the brain before the critical period, by implanting the brain into the abdomen, were successful in a few cases (3·6 %) i *Ephestia* (Kühn & Piepho, 1936) and in a greater proportion (15·3 %) in variou Sphingid larvae (Caspari & Plagge, 1935: Plagge, 1938). These implants wer successful only in larvae shortly before pupation; they were ineffective in larva 2–4 days after the last moult (Plagge, 1938).

Removal of the corpora allata in Orthoptera (*Melanophus* (Weed, 1936 *Dixippus* (Pflugfelder, 1937)) likewise does not prevent moulting. Though recentl Pflugfelder (1939) has induced two extra moults in adult *Dixippus* by the implantation of five or six corpora allata from young insects.

Among Diptera, on the other hand, there is good evidence that the hormone
ich induces pupation in flies (*Calliphora* (Fraenkel, 1935); *Drosophila* (Bodenstein,
36)) is secreted by the ring gland or Weismann's ring (Hadorn, 1937; Burtt, 1938),
d that this gland probably contains the homologues of the corpora allata and the
sophageal ganglia (Burtt, 1937, and unpublished work).

At the present time, therefore, there seems to be little uniformity as to the
urce of this hormone among the few insects studied.

As regards hormones acting more or less like the "inhibitory hormone" in
odnius: these have been clearly demonstrated as coming from the corpora allata
Lepidoptera by Bounhiol (1938) and by Piepho (unpublished experiments);
ugfelder's (1937) observations on the results of excision of the corpora allata in
ung *Dixippus* nymphs may be similarly interpreted; and there is some indication
at the ring gland in muscid larvae has a similar effect (Burtt, unpublished work).

Mode of action of the inhibitory hormone

The main object of this paper was to study the mode of action of the hormones
controlling the morphological change at metamorphosis. For the moment it will
well to consider only those characters (the form of the cuticle, the presence or
sence of melanin, etc.) which are determined by the actions of single cells, and
fer discussion of complex changes such as the elaboration of wings or external
nitalia which require the co-ordinated activity of many cells.

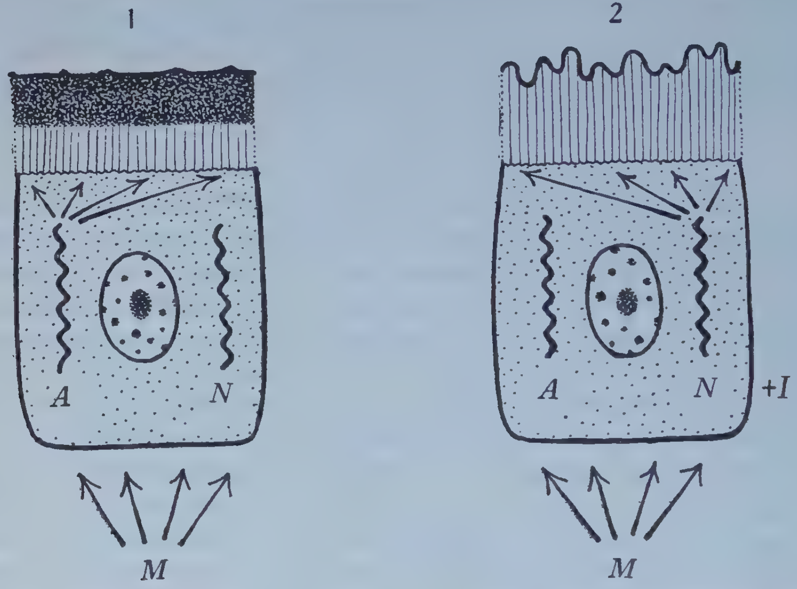
Now many of the cells persist from the nymph to the adult. The type of cuticle
ey lay down is dependent on the hormones present. Clearly each cell must contain
metabolic systems needed for the formation of both nymphal and imaginal
ticle. The role of the hormones is to determine which of these systems is effective.
nce we have no knowledge at present about the internal organization of the living
l, we can of course only speculate about the nature of these intracellular systems
ich it is necessary to postulate.

If the young nymph is decapitated at the critical period, it may show a switch
er in development from nymph to adult; and the cell (e.g. a bristle-forming cell)
y lay down a structure which is intermediate in form between that of nymph and
ult. Hence the two systems are not mutually exclusive—in many of the cells they
ust be very closely related chemically.

The setting in motion of these systems, the activation of the cell and the sub-
quent processes ending in the deposition of the new cuticle, is brought about by
moulting hormone. This may prove ultimately to be made up of more than one
nstituent; but for the moment it may be regarded as a single substance. When this
rmone alone is present, the system which leads to the deposition of the imaginal
ticle is caused to function. If the inhibitory hormone is added in varying amounts,
system which leads to the deposition of nymphal cuticle is activated to a
riable extent, and all grades of intermediate structures are produced.

Now it is characteristic of the inhibitory hormone that it causes more rapid
velopment than the moulting hormone alone (a 5th stage nymph at 24° C. will

moult to an adult in 28 days; it will make a nymphal moult in 17 days (Wiggles worth, 1934)). We may thus formulate an hypothesis for the control of metamor phosis in *Rhodnius* as follows (Text-fig. 12). Each cell contains two enzym systems, imaginal and nymphal. The moulting hormone initiates the process o growth and cuticle formation in the cell and when it alone is present the imagina system is activated and leads to the secretion of imaginal cuticle (Text-fig. 12, 1 The "inhibitory hormone" activates the nymphal system; it acts perhaps as "co-enzyme"; and since this system works more rapidly than the imaginal, th production of imaginal structures is suppressed[1] when this hormone is also presen (Text-fig. 12, 2). Even after the assumption of the adult form, the nymphal system must be supposed to remain intact within some of the cells at least. For when th adult is caused to moult this system may be activated once more and a nympha cuticle is laid down.



Text-fig. 12. Diagram to illustrate hypothesis for the control of metamorphosis in *Rhodnius* hormones. 1, formation of imaginal cuticle in presence of moulting hormone alone. 2, formation nymphal cuticle in presence of moulting hormone and inhibitory hormone. A, imaginal system; nymphal system; M, moulting hormone; I, inhibitory hormone.

This hypothesis is an elaboration of the idea, already put forward (Wiggles worth, 1936) that the determination of characters at metamorphosis results fro the interaction of two developmental processes with different velocities. Its ver close analogy with Goldschmidt's well-known theory of sex determination an intersex production is obvious. In the foregoing discussion it has been considere only from the point of view of single cells which persist from one stage to the nex ut the ideas require only slight modification to cover those cases where there is tensive cell death at metamorphosis and much change in form through the -operation of the dividing cells.

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[1] Suppression of the imaginal system may, of course, be brought about by other means; such the absorption by the nymphal system of some essential factor. This would bring the hypothesis in line with that put forward to explain the suppression of new plaques around existing plaqu (Wigglesworth, 1940).

In previous papers the "inhibitory hormone" was so called because in its esence the production of imaginal characters at moulting is suppressed. But in ew of its probable mode of action through the activation of the nymphal system the expense of the imaginal, it might be preferable to refer to this hormone as the ymphal" or "juvenile" hormone.

SUMMARY

Nymphs of *Rhodnius* decapitated 24 hr. after feeding can be induced to moult implanting into the abdomen the dorsal region of the protocerebrum removed om other nymphs during the critical period. Implantation of other parts of the ain, of the corpus allatum, and of fat body from the same insects did not cause oulting.

The presence of large nerve cells with fuchsinophil inclusions discovered by anström in this region of the brain has been confirmed.

The histological changes in the epidermis of the abdomen and the distribution mitoses at an imaginal moult and at a nymphal moult have been compared. uring a nymphal moult mitoses occur all over the tergites; during an imaginal oult they are largely confined to the intersegmental membranes and the periphery the abdomen, and there is a more extensive breakdown of existing cells.

If 5th stage nymphs in the course of moulting to become adults receive "inbitory hormone" from young nymphs, they may be caused to "switch over" to mphal development. Such a "switch over" soon becomes impossible for the ost specialized structures of the adult; other structures follow in turn; but the neral cuticle of the tergites may still be influenced up to a short time before it is ue to be laid down. The various faculties of a given cell can become determined to me extent independently of one another.

Isolated fragments of cuticle and epidermis from *Rhodnius* adults may be induced moult, more than once, by transplantation to young moulting nymphs.

Decapitated *Cimex* adults may be caused to moult again if they receive blood om moulting *Rhodnius* nymphs; but they lay down a normal cuticle with bristles ly if they have become adult very recently.

Decapitated *Rhodnius* adults may be caused to moult again if they receive blood om two moulting 5th stage nymphs. They lay down a cuticle of normal adult type en when they have been adults for several months. The old skin is digested up to e level of the exocuticle.

If such moulting adults are provided with inhibitory hormone from the corpus atum of young nymphs, they show a partial reversion to nymphal characters nen they moult. This change probably does not extend to the most specialized aginal structures; but the pigmentation and the structure of the general cuticle, d of the bristles it carries, may become partially nymphal again.

The "determination" of imaginal or nymphal characters thus takes place a different times in different organs. And for some characters, at least, such determination is not irrevocable. In the light of these results a new hypothesis is pu forward to explain the action of the inhibitory hormone in controlling metamorphosi

This work was begun during a stay of three months in Berlin-Dahlem as th guest of the Kaiser-Wilhelm Gesellschaft, to whom my thanks are due. Throughou this stay I was indebted to Prof. A. Kühn and his colleagues at the Kaiser-Wilhel Institut für Biologie for every assistance and for much stimulating discussion.

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EXPLANATION OF PLATE I

Tergites of insects produced from 5th stage nymphs switched over from imaginal to nymph development at different times after feeding.

Fig. 1. 5 days. Fig. 3. 9 days. Fig. 5. 12 days. Fig. 7. 15 days.
Fig. 2. 7 days. Fig. 4. 11 days. Fig. 6. 13 days. Fig. 8. 17 days.

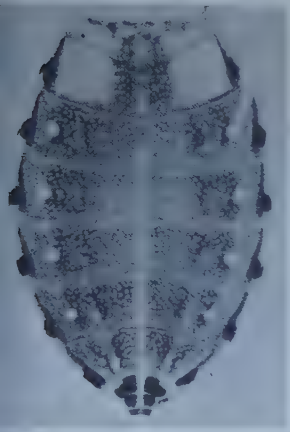


Fig. 1.

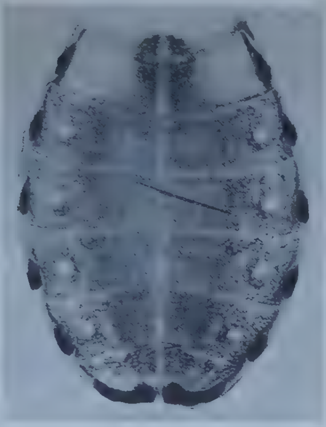


Fig. 2.

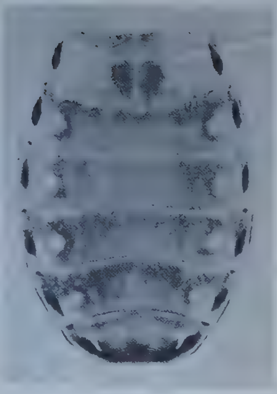
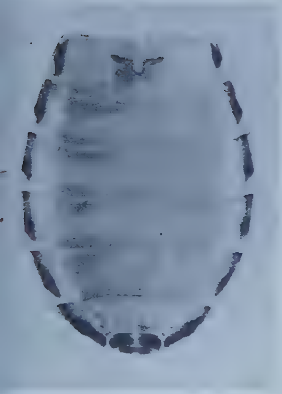


Fig. 3.



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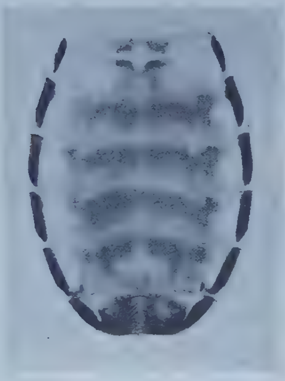

Fig. 5.

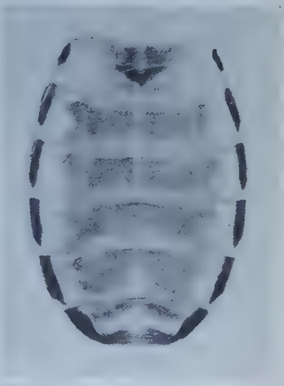

Fig. 6.

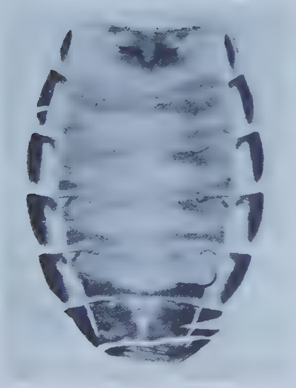

Fig. 7.

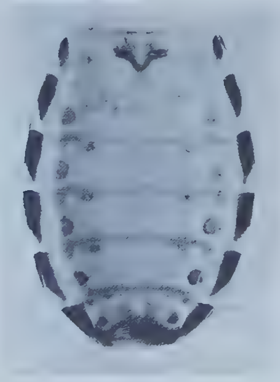

Fig. 8.

WIGGLESWORTH—THE DETERMINATION OF CHARACTERS AT METAMORPHOSIS IN *RHODNIUS PROLIXUS* (HEMIPTERA) (pp. 201–222)

A NOTE ON THE OSMOTIC PRESSURE OF THE BLOOD OF VARIOUS ANIMALS

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(*Received* 14 *December* 1939)

(With One Text-figure)

The experiments described below were undertaken originally in conjunction with Dr Dacie of King's College Hospital in an attempt to determine whether a relation exists between the osmotic pressure and the "fragility" of blood corpuscles. It was ound, however, that the osmotic pressure differences hardly accounted for the ragility differences, and the experiments were continued on the osmotic pressure lone.

Baldes's (1934) modification of A. V. Hill's (1930) vapour-pressure method was used. The thermocouples had very small loops and were constructed by Mr J. L. Parkinson of 47 s.w.g. (51μ) constantan and manganin wires. The wires were brazed together under a stereoscopic microscope and wound on a former to shape he loops. The couple was then insulated by coating with "Trolitrul" dissolved n benzole, each layer being allowed to dry before applying the next. After removal rom the former, two or three coatings of air-drying bakelite varnish ("yacht arnish") were applied and allowed to harden thoroughly under a lamp before the ouple was used.

The galvanometer was a highly sensitive moving-coil instrument (Zernicke Zc) ead at a distance of about three metres.

A drop of blood was placed in one loop, and a drop of the standard solution 1 % NaCl) in the other, by means of very fine pipettes which passed through the orks of the fluid containers. This operation was carried out in a damp chamber o prevent evaporation. The loops were immediately covered with a tube lined with lter paper soaked in the standard solution. The whole was then placed in a large vater thermostat for 10 min. to settle down. In the first 5 min. a mixture of 5 % CO_2 in oxygen was passed slowly through. The difference of vapour pressure etween blood and standard was calculated from the readings by calibrating the ouples with the known difference between two solutions. Three thermocouples vere used and double determinations were made with each; i.e. the blood was laced first in one loop and then in the other, and the mean taken, to eliminate rrors due to lack of symmetry.

Great precautions were taken in drawing the blood from the animal, for exertio considerably raises the osmotic pressure (Margaria, 1930). In the case of mammal the blood was drawn from a vein in the neck with the animal as passive as possible not having been chased and not struggling to get free. Blood from slaughte houses would be useless. Birds were wrapped in a cloth and kept quite quiet whil the blood was drawn from a vessel under the wing. With tortoises the head was cu off suddenly and the blood collected immediately. In all cases about 1 c.c. o blood was used and 0·2 mg. of heparin was added to prevent clotting. Specia experiments showed that this amount of heparin has no effect on the osmoti pressure.

The osmotic pressures are given in terms of the equivalent percentage of NaC (g. NaCl in 100 g. H_2O).

Table I. *Osmotic pressures of blood exposed to* 5 % CO_2 *in* O_2: *expressed in equivalent concentrations of* NaCl (*g. per* 100 *g.* H_2O)

Sheep	Six animals:	0·988, 0·971, 0·972, 0·974, 0·973, 0·988	Mean **0·978**
Goat	Five animals:	0·943, 0·957, 0·962, 0·958, 0·955	Mean **0·955**
Horse	Six animals:	0·900, 0·930, 0·922, 0·943, 0·926, 0·940	Mean **0·927**
Cattle	Eight animals:	0·858, 0·950, 0·952, 0·933, 0·921, 0·951, 0·921, 0·923	Mean (neglecting 0·858) **0·936**
Dog	Nine animals:	0·900, 0·947, 0·947, 0·965, 0·928, 0·938, 0·918, 0·931, 0·918	Mean **0·933**
Man (Margaria, 1930, for comparison):			Men **0·945** Women **0·927**
Pigeon	Six birds:	0·980, 1·002, 1·020, 1·025, 0·996, 0·999	Mean **1·004**
Hen	Six birds:	0·992, 1·000, 1·080, 0·997, 1·085, 0·994	Mean **1·025**
Tortoise	Seven animals:	0·808, 1·161, 0·991, 1·010, 1·024, 0·902, 0·805	Mean **0·957**
Frog (Hill & Kupalov, 1930, for comparison, but without CO_2):	Eleven animals:	0·762, 0·721, 0·723, 0·733, 0·784, 0·715, 0·739, 0·783, 0·705, 0·686, 0·688	Mean **0·731**

The results of the measurements on fifty-three animals are given in Table I The average deviation from their mean of the readings on one individual was abou 0·012, so the probable error of each value in the table is about 0·005. The variations therefore, between the different animals of each species are most probably genuin and not to be attributed to errors of observation. The values are shown graphicall in Fig. 1. All the values for sheep are higher than any value for goat. The value for horse, cattle and dog are not significantly different and are close to Margaria' (1930) values for man. The values for pigeon and hen are about the same as on another and considerably higher than for any mammal except sheep.

The values for tortoise are so widely scattered that the mean has little significanc he first four animals were obtained from a shop, and it was thought that the large ariations observed might be due to the conditions under which they had been kept. s shipped to this country they are without food and water and they are frequently ept at the dealers still without water. The last three animals were obtained by the indness of the Zoology Department of University College. These had been kept or some weeks with plenty of green food and water, but they gave large variations

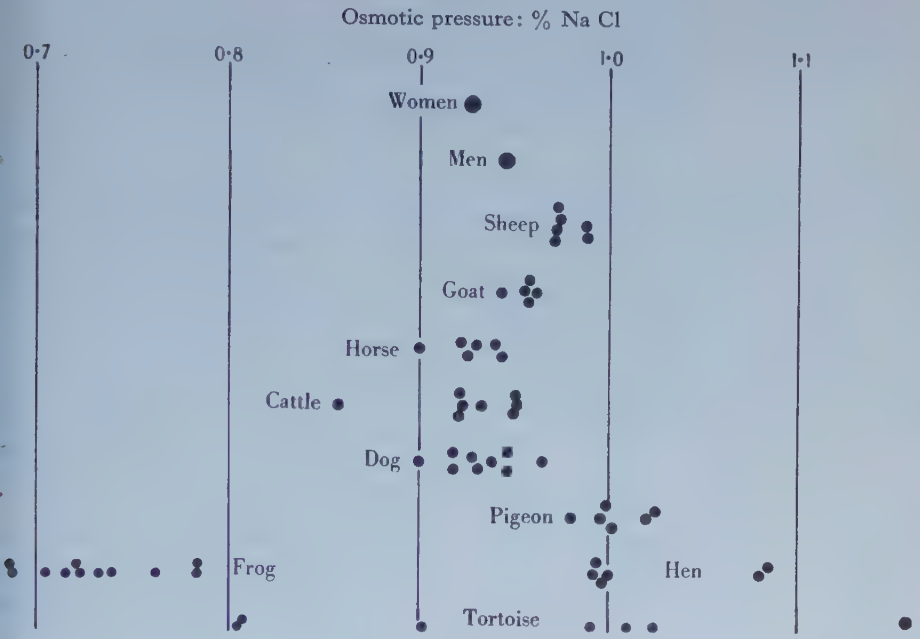

Fig. 1.

till, indeed the highest and the lowest of the seven. No simple explanation, herefore, can be given of the inconsistency, but it is very striking. The values for ortoise are far higher and far more variable than those for frog: indeed Hill and upalov's (1930) values for frog are nearly as consistent as those of the mammals nd birds in the present series.

I must thank Prof. A. V. Hill for his advice and interest and Mr J. L. Parkinson or his help and for constructing, insulating and testing the thermocouples. I am ndebted to the Royal Veterinary College where I obtained most of the blood amples.

SUMMARY

The osmotic pressures have been measured, using the vapour-pressure method, f the blood of six animals and two birds, sheep, goat, horse, cattle, dog, tortoise, igeon, hen. Precautions were taken to avoid the rise of osmotic pressure due to truggling or movement.

The mean value for sheep is appreciably higher, and that for goats slightly higher than for other mammals. Apart from sheep, the mammals have nearly the same value as man. The birds have a considerably higher value. In tortoises the value is very variable (0·80 to 1·16) and much higher than in frogs (0·73).

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THE EFFECT OF DEAFFERENTATION UPON THE LOCOMOTORY ACTIVITY OF AMPHIBIAN LIMBS

By J. GRAY and H. W. LISSMANN

Zoological Laboratory, Cambridge

(*Received* 1 *December* 1939)

(With One Plate and One Text-figure)

In 1912–13 Graham Brown and Sherrington showed, independently, that rhythmical activity could be maintained in a pair of deafferentated antagonistic mammalian muscles; since then it has been customary to regard locomotory rhythms as the expression of intrinsic activity within the nerve cord. On the other hand, a study of the responses of vertebrate limbs to suitable reflex stimulation makes it difficult to believe that impulses coming from peripheral sense organs do not play a very important role in the co-ordination of normal locomotory movements. The present paper constitutes an attempt to decide how far locomotion can occur in animals whose limbs have been completely desensitized by cutting the roots of their afferent nerves.

The work of Mott & Sherrington (1895), Bickel (1897) and Ranson (1931) shows that, after section of all its dorsal roots, a limb of a monkey, dog or cat fails to take part in subsequent locomotion. Such a profound effect of deafferentation in the limbs of otherwise intact mammals is in striking contrast to the observations of both Graham Brown (1912*a*, *b*, 1913) and Sherrington (1913) on isolated preparations of mammalian muscles; both these authors found it possible to elicit rhythmical stepping in a pair of deafferentated muscles so long as the nerve cord was exposed to an appropriate bilateral stream of afferent impulses.

The behaviour of deafferentated limbs in lower vertebrates has been described by various investigators. Trendelenburg (1906) found that the power of flight of a pigeon was not seriously disturbed by deafferentation of one wing; if, however, both wings were desensitized the bird could not fly. In amphibia, both Hering (1897) and Bickel (1897) found that deafferentation of one or both hindlimbs of the frog did not seriously impede swimming or jumping. Deafferentation of all four limbs reduced locomotory activity to a very low level (Bickel, 1897), and after total deafferentation of all spinal nerves no co-ordinated movements were observed by Hering (1893). Within recent years, Weiss[1] (1936) has confirmed the fact that deafferentation of both hindlimbs of a toad (*Bufo americana* or *B. fowleri*) does not prevent these limbs from displaying their normal locomotory activity. The description, given by Weiss, of a toad deafferentated in all four limbs does not differ substantially from that given by Hering (1897), but, nevertheless, Weiss concluded that: "However serious the effect of deafferentation may be with respect to the total behaviour of the animals, it certainly cannot be said to involve a disintegration of the basic patterns of motor co-ordination as such; once made to appear, these manifest themselves in—qualitatively speaking—full integrity."

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[1] Our attention was only drawn to this paper after our own observations were complete.

It is important to note that none of the above authors gives more than somewhat casual reference to the effect of deafferentation on ambulation: they were chiefly concerned with jumping and swimming, wherein the forelimbs play only a minor role and in which the movements of the hindlimbs are simultaneous.

During a study of the flexor reflexes of the limbs of spinal amphibia we have found it difficult to avoid the conclusion that peripheral events play a very important role in the co-ordination of the terrestrial locomotion of these animals, and it therefore seemed of interest to consider in some detail how far typical terrestrial movements can be displayed after deafferentation of the limbs. For these experiments we have used the toad (*B. bufo*). This animal seldom jumps unless violently stimulated; it walks with a characteristic diagonal sequence of limb movement (see Gray, 1939). It is with this pattern of movement that the present paper is concerned.

The nerve supply to the limbs of *Bufo* is essentially the same as that of *Rana*. The main supply to the forelimbs is from spinal nerves III and IV; V, VI and VII supply the trunk of the body; VIII, IX and X supply the hindlimb; and nerve XI supplies a small area in the neighbourhood of the anus. A small branch of the hypoglossal is also associated with the forelimb. Severance of this nerve caused respiratory disturbances, and animals, so operated upon, did not survive well. For this reason, the hypoglossal was usually left intact. Its sensory fibres did not appear to be associated with sense organs in the forelimb, since severance of nerves III and IV caused complete loss of sensitivity—both superficial and deep seated—in the limb. All operations were performed under ether, and the animals were kept under observation for periods varying from 2 days to 3 weeks. In no case did the pattern of behaviour change after the lapse of time.

DEAFFERENTATION OF SINGLE LIMBS

After deafferentation of one hindlimb, it is not easy to differentiate between the normal and the operated limbs. Ambulation can be elicited by stimuli apparently no more intense than that required by a normal animal, and the characteristic diagonal pattern of movement is retained. When at rest, the animal adopts its customary squatting attitude, although in some cases there appeared to be a tendency to hold the plantar surface of the operated limb off the ground as was observed by Hering (1897). The operated limb is entirely anaesthete and offers no resistance to external passive displacement, but as soon as the animal moves (in response to suitable visual, labyrinthine or mechanical stimulation), the limb quickly takes up its proper position and functions in a normal manner.

After deafferentation of one forelimb little or no disturbance of ambulatory behaviour can be detected when the animal is moving relatively rapidly. During slow movement, however, a loss of tone can be observed in the muscles of the orearm and digits; the forearm may rest on the ground during the retractor[1] phase of the upper arm's movement, and the digits are not always extended. When he animal is at rest, the posture of a deafferentated limb is sometimes normal, but, not infrequently, the forearm rests on the ground, causing a downward displacement of the body towards the operated side. So far as could be seen, deafferentation of a single limb only disturbs the rhythm of locomotion in so far as loss of tone in he wrist involves purely mechanical effects.

Some of the features observed in the deafferentated forelimb of a toad, recall he observations of Mott & Sherrington (1895) on the monkey (*Macacus rhesus*), viz. the loss of muscular tone, and the impairment of movement in the distal joints of the limb. On the other hand, whereas the deafferentated limb of the monkey, dog (Bickel, 1897) or cat (Ranson, 1931) plays no part in ambulation, that of the toad continues, particularly in its upper arm or thigh, to play its normal role in diagonal ambulation.

It should be noted that the ability of a single deafferentated limb to take its normal part in ambulation does not constitute evidence in favour of the central nature of the ambulatory rhythm; ample evidence exists to show that stimulation of the peripheral sense organs in one limb can induce co-ordinated responses in neighbouring limbs.

DEAFFERENTATION OF TWO LIMBS

The effect of deafferentation of two limbs depends somewhat on the limbs selected for operation, but, in general, our observations confirm those of Weiss (1936) in demonstrating that, after deafferentation of two limbs, the locomotory pattern is singularly little disturbed.

If both hindlimbs are deafferentated, the animal walks with the usual diagonal sequence of limb movements but displays the following abnormalities: (i) the hind region of the body is not lifted off the ground to the normal extent, and (ii) the femur s not infrequently protracted to an abnormal extent. These features seem to be due to a loss of tone by the muscles rather than to a functional disturbance of the ocomotory rhythm.

Deafferentation of one hind- and one forelimb did not seriously impede ocomotory activity, but no records were kept of minor disturbances. After deafferentation of both forelimbs, all operated animals displayed characteristic features. In no case was the pattern of ambulation seriously disturbed, but the ollowing minor abnormalities were present to a varying extent:

(i) Both at rest and during retraction of the humerus, the whole of the forearm was kept in contact with the ground, whereas in the normal animal the forearm is aised.

(ii) The degree of protraction of the humerus was abnormally great; at the moment of maximum protraction the upper arm faced obliquely forwards instead f lying at right angles to the axis of the body as in the normal animal.

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[1] The term *retraction* is used to denote the movement of the whole limb during the propulsive hase of the step; *protraction* denotes the non-propulsive phase.

(iii) The amplitude of step of the hindlimbs appeared to be greater than normal

The first two of these features prevent the forelimb from functioning as an efficient prop on which the body can be swung forward during its own retractor movement and that of the diagonal hindlimb; the animal thus tends to roll somewhat and the smoothness of progression is diminished. In no case, however, was the normal diagonal pattern of movement lost.

At this point, it is of interest to note the results obtained by deafferentation of both forelimbs of the newt (*Triton cristatus*). Such animals, when at rest, held the forelimbs in a partially retracted position not observable in intact animals; the plantar surfaces were held off the ground and the elbows pointed obliquely backwards. When the animals walked slowly, the forelimbs showed no movements—the body being pressed forwards by the hindlimbs and body muscles only. When, however, the animals were stimulated into rapid movement, by tapping on the tail, the forelimbs at once took their usual part in locomotion—and showed typical diagonal sequence with the hindlimbs. As soon as progression ceased, the retracted posture of the forelimbs was resumed. The behaviour of these limbs is interesting in view of the observations of Detwiler & Vandyke (1934); these authors found that no locomotory movements occurred in the forelimbs of a larval *Amblystoma* if the development of afferent nerves had been prevented by destruction of the dorsal crest of the nerve cord in an early stage of development.

The mobility of the deafferentated forelimbs of these newts when exposed to relatively strong stimulation recalls a similar phenomenon observed by Mott & Sherrington (1895); forcible and rapid movements occurred in the apaesthete limb of a monkey when the animal was induced to "struggle".

DEAFFERENTATION OF THREE LIMBS

Deafferentation of three limbs involves a still further loss of muscular tone and a marked decrease in the excitability of the animal. In such animals the body is not lifted from the ground either during periods of rest or during locomotion; the movements of the limbs are spasmodic in the sense that the retractor phase sets in abruptly. The diagonal sequence of limb movement, although not invariably preserved, is nevertheless clearly present, particularly in more active preparations. The animals usually only walked in response to fairly strong tactile stimuli, but if left in a lighted tank they walked spontaneously, after a fairly long latent period, towards a shaded area. Periods of active locomotion were brief, the animal fatigued quickly, movements were often confined to the shoulder in the forearm and to the thigh and knee in the hindlimb.

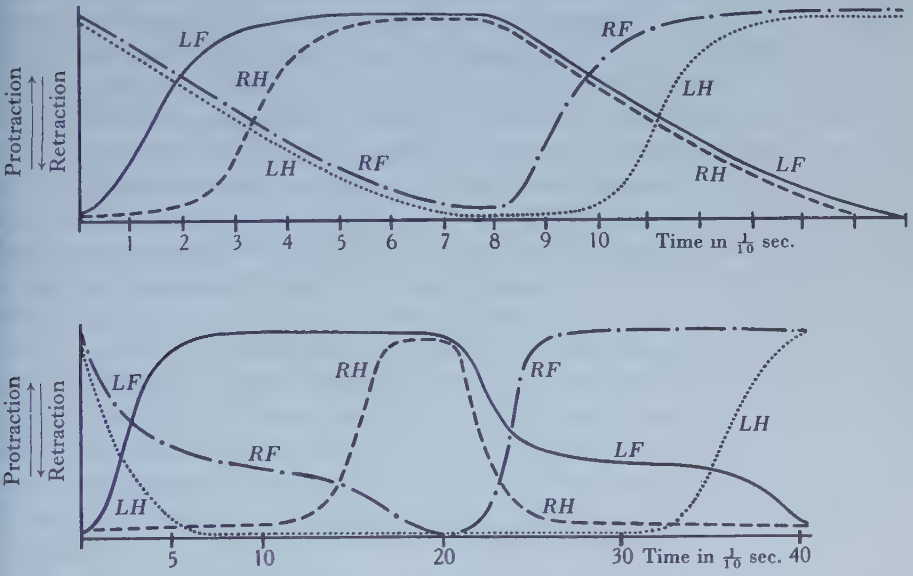
DEAFFERENTATION OF FOUR LIMBS

Deafferentation of all four limbs involves a marked decline in the excitability of the animal, and relatively strong stimuli, applied to the skin of the body, are required for the elicitation of limb movements; in many cases these movements re unco-ordinated. The ability of the animals to walk normally was best observed y placing the animal in a lighted tank and allowing it to walk spontaneously towards darkened area. Under such conditions, clearly defined diagonal co-ordination was bserved, but in every case the limb movements were ungainly, slow and few in umber. The chief abnormalities observed were:

(i) Considerable slip occurred during the retractor phase. The limbs were quickly xtended to their maximum extent, and there was often a long delay before the nset of contraction in the protractor muscles.

(ii) The body was not lifted off the ground during the retractor phase, but ended to roll from side to side owing to weakness of the forelimbs.

(iii) The hindlimbs were not infrequently extended somewhat laterally instead f posteriorly as in the normal animal.



ext-fig. 1. Reconstructions (from cinematograph records) of a relatively rapid step by a normal oad (A) and of a toad (B) deafferentated in all four limbs. In the normal animal protraction of a orelimb is accompanied by simultaneous retraction of the ipsilateral hindlimb, and the period lapsing before protraction of the contralateral hindlimb is short. After deafferentation co-ordination maintained with the ipsilateral hindlimb, but there is a long latent period before the contrateral hindlimb responds by retraction.

(iv) The smooth rhythm of movement of the normal animal is entirely absent; ach limb movement being spasmodic.

(v) Abnormal limb postures were frequently observed, both hindlimbs exhibiting, t times, a state of maximum retraction (see Pl. I, fig. D 2, 4). This abnormality an be traced to a very long latent period between the protraction of a forelimb nd the corresponding phase in the contralateral hindlimb (see Text-fig. 1 B).

Some, at least, of these characteristics, appear to be due to a lack of muscular one particularly in the more distal regions of the limbs. Very rarely did such animals progress for more than one or two co-ordinated steps at a time. Fatigue set in very rapidly.

The behaviour of the forelimbs of these animals only differed in minor respects from that of animals in which the forelimbs alone were deafferentated: the frequency of movement was much lower and the loss of muscular tone was perhaps more obvious. There could be no doubt, however, that deafferentation of the forelimbs had a profound effect upon the behaviour of deafferentated hindlimbs. The relatively smooth rhythm of movement is lost, the posture of the limbs is often abnormal, and the threshold stimulus for locomotion is very high. As noted by Weiss "the effect of deafferentating all limbs is immensely greater than one would expect from simply adding up the effect of fore- and hindlimb deafferentation".

At first sight, the ability of these toads to walk normally, even if only for a few slow and somewhat modified steps, tends to support the view that the locomotory rhythm is essentially of central origin, and that such irregularities as occur are due to general lack of muscular tone or lack of excitability in the motor centres of the limb muscles. On the other hand, the very marked difference in the activity of deafferentated hindlimbs before and after deafferentation of the forelimbs suggests that the hindlimbs are definitely influenced by peripheral events in the forelimbs and therefore also, possibly, by similar events in other parts of the body. Since the total ambulatory activity of an animal is reduced to such a low level by deafferentation of all four limbs, it is important to consider how far the remaining remnant of co-ordinated movement can be reasonably attributed to the presence of locomotory musculature not deafferentated by severance of the dorsal roots of nerves III, IV, VIII–XI (inclusive). No evidence of such musculature could be found in respect of the hindlimbs by direct stimulation of the body muscles, but it is possible, by such methods, to show that contraction of the anterior regions of the abdominal recti muscles causes retraction of the humerus by pulling on the posterior region of the large pectoralis muscle. Since the abdominal recti muscles are supplied by the Vth and VIth spinal nerves, it is of interest to consider how far complete deafferentation of all the spinal nerves still further reduces ambulatory activity.

TOTAL DEAFFERENTATION OF ALL SPINAL SEGMENTS

In six animals, all limbs and all the intervening segments were deafferentated by cutting the dorsal roots of spinal nerves III–XI (inclusive) on both sides. In no case did spontaneous co-ordinated ambulation occur, nor could it be elicited either through the eyes or through the labyrinth. If the body was rolled to the left, the left hindleg was actively extended: when placed on a turn-table and rotated to the right the right hindlimb was alternately flexed and extended—it failed to rotate the body owing to the inability of the planta to maintain contact with the ground. No trace of diagonal ambulatory co-ordination was ever observed. Apart from cleaning reflexes, only two types of co-ordination in the limb movements of these animals were observed:

(i) A few, but active, swimming movements were sometimes seen in the hind imbs when the animal was placed in water: both hindlimbs flexed simultaneously nd extended simultaneously.

(ii) If a moving object was suddenly brought near the eyes or if the head was ently tapped with a seeker, all four limbs moved in a purposive manner; the hindimbs were brought into the fully flexed position; the head was lowered and the orelimbs brought close up to the sides of the head.

Weiss (1936) described the behaviour of one animal of this type and concluded hat its behaviour was essentially the same as one in which the dorsal roots of the pinal nerves V, VI and VII were intact. "Occasionally the animals move for a ew steps with the hindlimbs alternating as in walking" (Weiss). In our experiments, ve failed to detect any pattern of limb movement which appeared to be of ambulatory ignificance unless at least one dorsal root was intact.

Since the integrity of one dorsal spinal root appears to be essential for the naintenance of the ambulatory pattern it is of interest to consider how far the sense rgans associated with this nerve are acting as essential pacemakers for the rhythm r how far they are concerned with the maintenance of a stream of impulses which nables the nerve cord to generate its own efferent rhythm. If the presence of roprioceptor sense organs in the muscles or skin enervated by nerves V, VI and II are acting as pacemakers for the ambulation of a toad (deafferentated in all four imbs) ambulation should not occur if the ventral roots of these nerves are cut—eaving their dorsal roots intact. If, on the other hand, the dorsal roots are mainaining the general level of excitability of the cord, it might be possible to cut these entral roots and elicit ambulation by continuous tactile stimulation of the skin of he body. Four such preparations have been made, and in no case was ambulation bserved; stimulation of the skin or body wall only elicited cleaning responses or ompletely unco-ordinated limb movements. Either the proprioceptor endings re acting as pacemakers or they alone provide the type of stimulation necessary to naintain the locomotory activities of the nerve cord.

DISCUSSION

Three facts are clearly established by the present series of experiments: i) A comparatively large amount of the total locomotory musculature of the toad an be deafferentated without seriously disturbing the role which the deafferentated nuscles play in the maintenance of the normal ambulatory pattern. (ii) Deafferentaion involves a loss of muscular tone, and, if widespread, causes a marked increase n the threshold of stimulation which is required to elicit muscular responses in the imbs. (iii) If the whole of the locomotory musculature be deafferentated, the nimal can no longer walk. The simplest interpretation of these facts is to assume hat the ambulatory rhythm is essentially dependent upon the activity of peripheral ense organs in the muscles. The experiments provide no positive evidence in upport of the central control of amphibian ambulation; on the other hand, it cannot e regarded as disproved. After severance of all afferent spinal nerves, III–XI (inclusive), the limbs of a toad will still respond by alternating flexion and extension to labyrinthine stimulation; the animal can sometimes swim, but it cannot walk.

The ambulatory activity of a single totally deafferentated limb of an amphibian forms, at first sight, a marked contrast to the immobility of a similar limb in a mammal. It is, however, interesting to note that Mott & Sherrington found that quite extensive deafferentation could be carried out on a monkey's limb before marked impairment of movement was observable. To some extent this was attributed to an overlap of sensory areas, but even when a field of complete anaesthesia was established on the most sensitive parts of the limb the impairment was comparatively slight. Even in mammals, therefore, a deafferentated muscle may prove to be sustainable in its normal movements by the influence of neighbouring intact musculature and skin. In the amphibia peripheral control can be exercised by sense organs lying in other limbs; in the mammal such control may be limited to sense organs lying within the operated limb. In this case the difference between the two types is quantitative rather than essentially different in nature.

In one respect the totally deafferentated limb of a mammal differs from that of an amphibian. When such a limb of a toad is not actively engaged in ambulation, its muscles appear to be entirely inactive, it is only during active movements that the limb displays muscular activity. The deafferentated limb of a mammal usually shows definite signs of muscular tone (Mott & Sherrington, 1895; Ranson, 1931). Ranson has described the behaviour of the deafferentated forelimb of a decerebrate cat as follows: "In the periods of activity which would come on spontaneously after brief periods of rest, the three normal limbs would show rhythmically repeated and well-co-ordinated movements in which the deafferentated leg did not as a rule participate. Instead, it would be rigidly extended and often drawn back against the chest wall. This posture of rigid extension would continue throughout the period of activity. In the intervals between these periods of activity, the deafferentated legs would relax and become considerably less stiff than the normal legs" (Ranson, 1931, pp. 342–3). The impression given by this account is that the extensor activity in an intact walking limb is counteracted at the appropriate moment by flexor activity induced proprioceptively within the limb itself. If the rhythm of ambulation were of central origin one would have expected the rhythm to appear even more readily in a deafferentated limb than in a normal limb.

So far as is known, there is only one series of facts which provides positive evidence in favour of the view that the ambulatory rhythm is of central origin. Both Graham Brown (1912*a*, *b*, 1913) and Sherrington (1913) showed that deafferentated pairs of antagonistic muscles would step if subjected simultaneously to two well-balanced but opposing streams of reflex stimuli. The crucial case is that given by Graham Brown (1912*a*), in which stepping was elicited from the deafferentated tibialis anticus and gastrocnemius muscles of a cat by severance of the nerve cord, for in this particular preparation all afferent nerves in the preparation had been cut; in Sherrington's preparations, the isolation of the nerve cord from proprioceptor influence was apparently not quite so complete. These rhythms must be of central origin, as are the cleaning reflex or swimming rhythms of a frog or toad. If, n the other hand, they represent true ambulatory movements, it is surprising that he deafferentated limb of the mammal does not participate in locomotion. Both Graham Brown and Sherrington noted that stepping only occurred in a pair of eafferentated muscles when the two opposing stimuli were very carefully balanced, ny excess from one side resulted in sustained contraction of one muscle and ustained relaxation of the other; excess of stimulation from the other side reversed he picture. Since the deafferentated mammalian limb does not step but appears o show sustained extensor activity, it would seem that the balance of opposing timuli is upset by deafferentation. In other words the balance is normally sustained y proprioceptor activity within the limb itself; in this case the gap between the entral and peripheral conceptions of the ambulatory rhythm seems narrower.

The ability of a toad to walk after extensive, but not complete, deafferentation ecalls a similar phenomenon in the swimming activities of a leech. So long as a ew segments of *Hirudo medicinalis* retain their nerve supply and are performing ctive swimming movements, relatively distantly situated efferent nerves (previously evered from their own musculature) display an electrical rhythm whose frequency s identical to that of the mechanical rhythm of the intact segments (Gray, Lissmann z Pumphrey, 1938). The electrical rhythmicity in such a nerve is at once abolished y severance of the nerve cord between the recording nerve and the intact regions f the body. As in the toad, it is possible to assume that a rhythm can be maintained n all segments of the body so long as peripheral pacemakers are operative in a few ntact segments.

SUMMARY

1. Deafferentation of not more than two limbs in the toad (*Bufo bufo*) only isturbs the normal diagonal pattern of ambulation in so far as a loss of muscular one in the operated limbs introduces complications of a purely mechanical nature. n some cases the extent and smoothness of the protractor and retractor phases of he step may be modified.

2. Deafferentation of three or four limbs involves marked loss of muscular tone, nd the excitability of the animal is greatly reduced. In some preparations the iagonal pattern of ambulation is preserved, in others it becomes irregular owing o a long latent period between the protraction of a forelimb and that of the contra-ateral hindlimb. Ambulation is ungainly, and fatigue rapidly sets in.

3. After total deafferentation of the whole body no ambulation has been bserved. No ambulatory activity was observed in preparations in which all four imbs were deafferentated and in which the ventral, but not dorsal roots, of nerves , VI, and VII were cut.

4. No positive evidence was found in favour of the view that the ambulatory hythm originates in the central nervous system. Ambulation only occurs when the fferent and efferent nerve supply of at least one spinal segment is intact.

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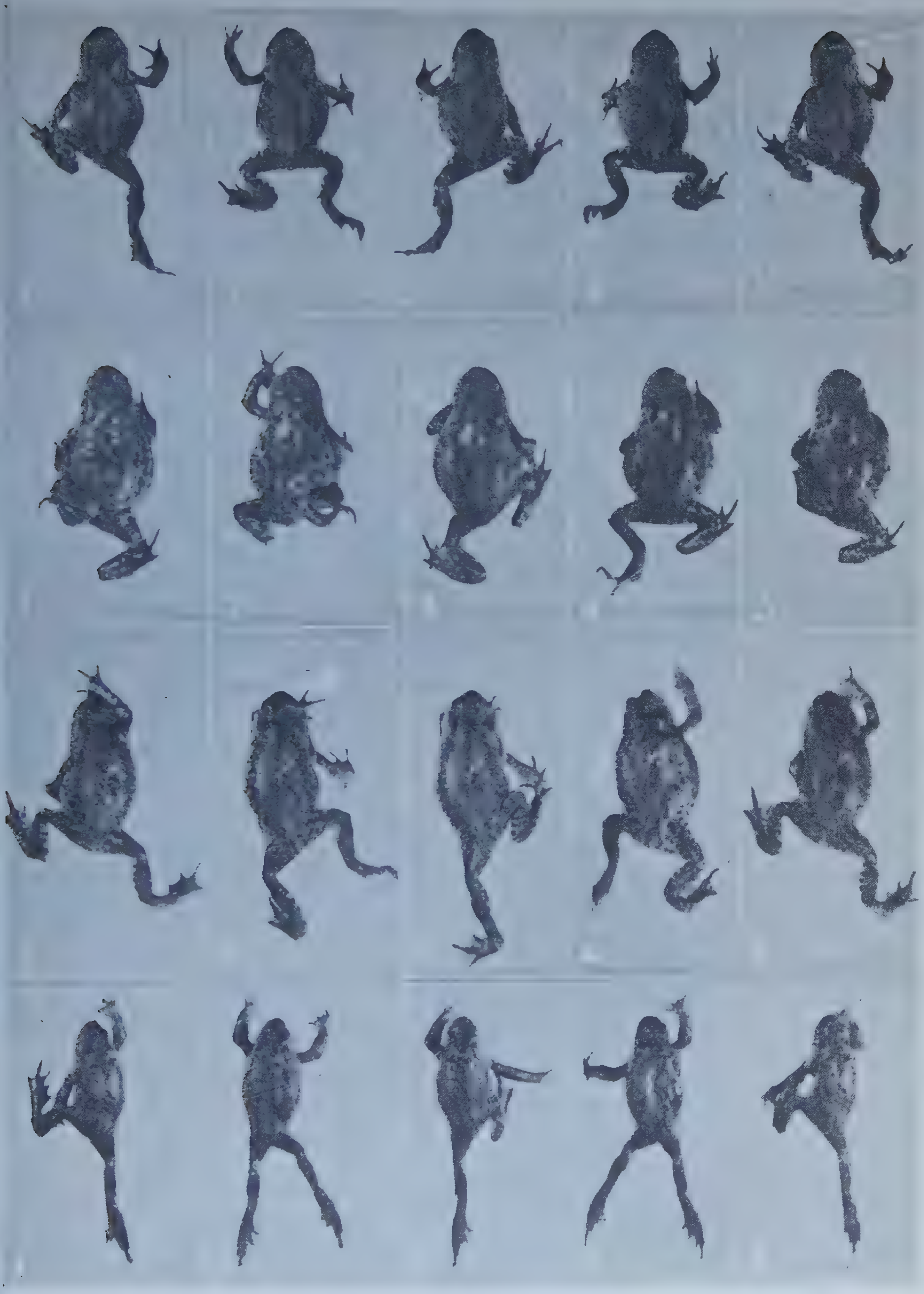
EXPLANATION OF PLATE I

Corresponding phases in the step of A, intact toad; B, toad after deafferentation of both hindlimbs C, toad after deafferentation of both forelimbs; D, toad after deafferentation of all four limbs [This plate is republished by permission of the Royal Society.]



GRAY AND LISSMANN—THE EFFECT OF DEAFFERENTATION UPON THE LOCOMOTORY ACTIVITY OF AMPHIBIAN LIMBS (pp. 227–236)

AMBULATORY REFLEXES IN SPINAL AMPHIBIANS

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(With Ten Text-figures)

HE profound effect of spinal deafferentation upon the locomotory activity of mphibians (Gray & Lissmann, 1940) suggests that peripheral sense organs play n essential role in the maintenance of normal ambulatory activity. In order to test his conclusion, a preliminary study has been made of those reflex responses which an be elicited from the limbs of spinal newts, frogs and toads, by the application of eripheral stimuli similar in kind and intensity to those which arise automatically uring normal progression. Unless otherwise stated, all observations were made on reparations in which the nerve cord had been severed at or about the level of the rst vertebra. All operations were performed under ether, and the preparations were llowed to recover from shock. The animals employed were *Triton cristatus*, *Rana emporaria* and *Bufo bufo*.

Before considering the behaviour of spinal preparations it is convenient to have relatively clear picture of the sequence of limb movements of a normal intact mphibian when walking, without change of direction, over a level surface. Under ich conditions the order of protraction[1] of the limbs exhibits the clearly defined iagonal pattern seen in most tetrapods, viz. left forefoot, right hind, right fore, left ind. Protraction of a limb invariably occurs during the retraction phase of (*a*) the ellow limb on the opposite side of the body, and (*b*) the other ipsilateral limb. uring relatively rapid progression, the duration of the protractor phase of the ewt's limb is approximately equal to that of the retractor phase and the two embers of each diagonal pair move simultaneously and in phase with each other; he start of retraction of a hindlimb is almost simultaneous with the onset of pro-raction of the ipsilateral forelimb. During slower progression, both phases of the step are slower, but the duration of the retractor phase is increased relatively to that of the protractor phase so that all four limbs are on the ground together at a definite though transitory phase of the step. This pattern of limb movement allows each limb to be protracted whilst the remaining three not only provide a stable support for the body but also, at the same time, propel the body forwards; when the speed of movement increases, two feet only are in contact with the ground for most of the stride and the triangular static type of support is replaced by the dynamically stable type provided by one pair of diagonal limbs.

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[1] The terms *retraction* and *protraction* are employed in this paper to cover, respectively, the whole ries of muscular movements involved during the propulsive and non-propulsive phases of the ep. They may be regarded as synonymous with the terms *extension* and *flexion* as these terms appear be used by some mammalian physiologists, but, as pointed out by Philippson, the final stages of e non-propulsive movement in a mammal involves the extension of the distal joints of the limb, hile flexion of these joints occurs during the propulsive movement. During the propulsive phase the forelimb of an amphibian the humerus is *adducted*, and is abducted during the non-propulsive hase; in the hindlimb the opening phase of the propulsive movement carries the axis of femur vay from the median line of the body, and the movement is largely in a plane at right angles to at occupied, during the propulsive phase, by the hindlimb of a mammal.

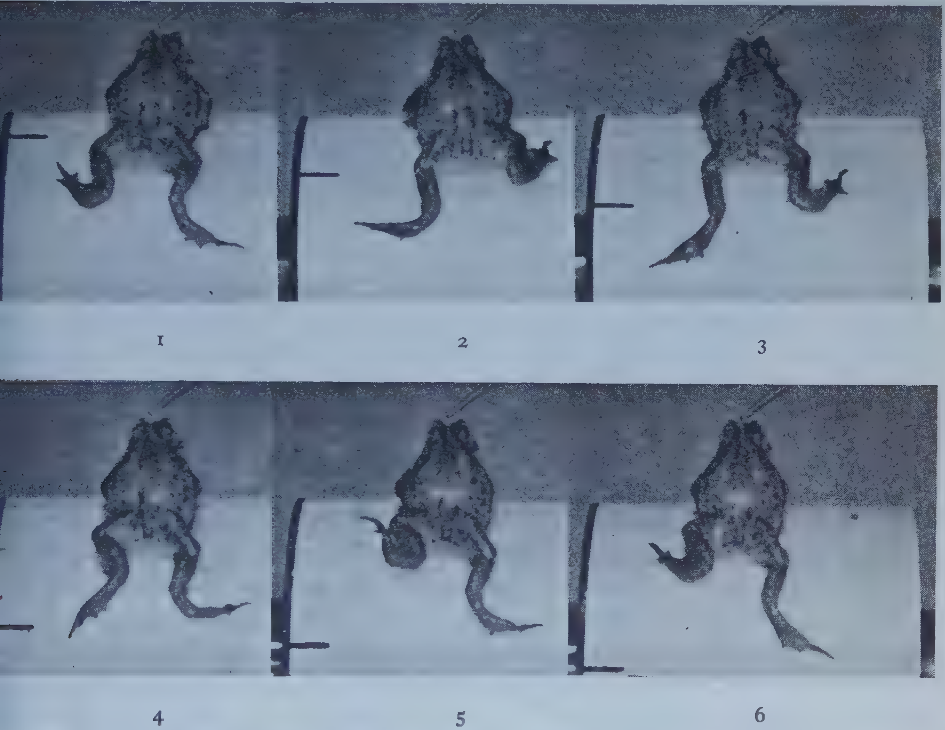
During each complete ambulatory cycle the animal is subjected to rhythms of tactile and proprioceptive stimulation, to both of which Philippson (1905) attributed essential roles in the co-ordination of normal walking in the dog. This conclusion and the objections raised to it by Graham Brown (1912 *a*, *b*, 1913) and Sherrington (1913) will be considered later, but it may be mentioned that we have, as yet, been unable to observe any clearly defined response by the spinal amphibian limb to tactile stimulation of the type observed in mammals by Philippson; on the other hand, stretch reflexes can be elicited in a regular and unmistakable manner. Before describing these reflexes it is convenient to consider the general picture presented by spinal preparations of amphibia.

Severance of the nerve cord at the level of the first vertebra induces three main changes in the activity of amphibia: (*a*) the power of spontaneous locomotion is completely and permanently lost, (*b*) reflex activity can be readily induced in the protractor muscles of the limbs, (*c*) relatively little reflex activity can be elicited from the retractor muscles. The picture presented by the newt, toad or frog is, in these respects, the same; but in one respect the urodele differs from the anurans. In *Triton*, the limbs of the undisturbed spinal preparation do not exhibit any characteristic posture, for each limb can be placed passively in a variety of positions; in *Rana* and *Bufo*, however, the hindlimbs of the undisturbed spinal animal are typically protracted so long as the posterior surface of the abdomen is in contact with the ground. If a spinal frog or toad be held vertically in air both limbs normally extend, and although they may exhibit somewhat irregular rhythmical movements no signs of regular or persistent stepping has been observed. In this respect the spinal amphibian appears to differ from the corresponding mammalian preparation.

During normal locomotion of an intact amphibian, protraction of a limb occurs when it is fully extended by the extensor muscles and it is therefore significant to note that, in the spinal animal, a limb invariably swings forward to the position characteristic of normal protraction whenever it is placed passively in the position of full retraction. This response is highly characteristic and extremely constant in occurrence.

The present paper is largely based on the striking observation that if all four limbs be simultaneously retracted, by passive stretch, they do not respond simultaneously or at random but in the definite sequence characteristic of the diagonal pattern so characteristic of ambulation in a normal intact animal.

In order to retract the limbs without exposing them to types of stimulation which do not arise during normal ambulation it has been found useful to place the animal the surface of a horizontal kymograph drum (Fig. 1). The surface of the drum s covered by a sheet of damp paper and the drum was revolved at a rate compar- le to, or less than, the normal walking speed of an intact animal.



. 1. Photographs showing one complete step by the hindlimbs of a spinal toad when the limbs in contact with the surface of a revolving drum. Period of step 1·7 sec. The preparation is pended at the anterior end and the posterior part of the abdomen rests on the drum.

RESPONSE OF SINGLE LIMBS TO PASSIVE RETRACTION

The response of single limbs to passive retraction can be observed by placing h of the three other limbs on a fixed support and allowing the planta of the fourth b to rest on the moving surface of the drum. Under such circumstances the limb contact with the drum exhibits, for prolonged periods of time, a series of well- ined protractor responses, the limb swinging actively forwards as soon as it is ved passively to the retracted position. These responses are very easily elicited m the hind limbs and have invariably been observed in all but moribund parations of *Triton*, *Rana* or *Bufo*. In the forelimbs, the response is less certain t, when it occurs, it is well-defined (see footnote, p. 242).

In *Triton*, the protractor response to passive retraction is of particular interest for s identical in every detail with the movement executed by the limb during the active traction phase of normal locomotion of an intact animal; the limb is lifted from l replaced on the ground with remarkable precision. (See Gray, 1939, Plate 6).

Response to passive extension is at once abolished by severing the dorsal roo of the nerves supplying the limb and there can be little doubt that excitation of th proprioceptor elements in the limb and its associated structures is an essential pa of the reflex mechanism.[1]

RESPONSE TO SIMULTANEOUS PASSIVE RETRACTION OF BOTH HINDLIMBS

When both hindlimbs are in contact with a moving surface, and both fore fe are resting on a stationary support, the hindlimbs "step" alternately with ea

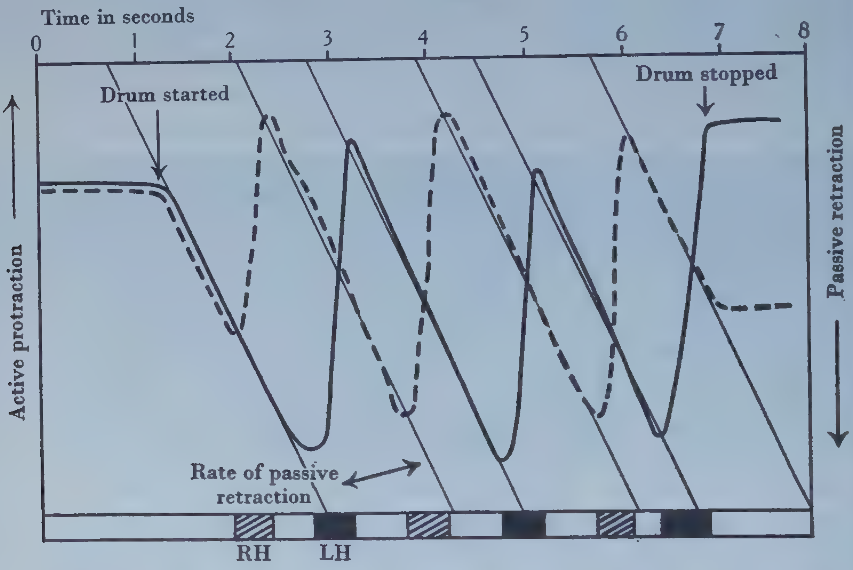


Fig. 2. Alternate stepping by hindlimbs of a spinal toad when the limbs are in contact wit revolving drum. Note the short initial step by the right limb and the cessation of stepping when drum ceased to move. The periods occupied by the protraction phase of each limb are shown the bottom of the figure. Graph derived from cinematograph record taken at 16 frames per seco The dotted line shows the movements of the right hindlimb, the full line shows the movements the left hindlimb.

other (Fig. 1). This phenomenon is very striking in all the species of amphibian e amined and was probably first observed by Bickel (1900) who recorded moveme in the hind-legs when frogs (whose nerve cords had been severed behind t brachial plexus) moved forwards spontaneously owing to active movements e forelimbs. Similar observations were made on urodeles by Snyder (1904) and en Cate (1928).

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[1] Passive retraction also sensitizes the protractor centres of the limb to stimulation arising other regions of the body. This is particularly clear in *Triton*: if a hindlimb be placed passiv near to, but not at, the position of maximum retraction, a gentle tap on the tail from a blunt instrum will induce protraction of the limb. If, on the other hand, the limb be placed passively in position of full protraction, and a series of taps be applied to the tail, the limb actively exte until, on reaching the position of full protraction, it automatically retracts. In this way a limb be kept stepping for a considerable time. It is clear that the response of the newt's limb to extrane stimulation depends on the passive posture of the limb at the moment of application of the stimulu as is the case in the dog (Magnus, 1909).

We have made a large number of observations on the stepping activities of pairs limbs, all of which illustrate very clearly that protraction does not occur simul- neously. When the hindlimbs of a spinal toad or frog are placed on a stationary um both limbs remain protracted as long as the ventral surface of the body is in ntact with a stationary drum. When the drum is set in motion, both limbs begin to tend passively, and after a short initial step by one limb, protraction occurs ernatively in each limb whenever its position of full retraction is reached (Fig. 2). he short initial step is possibly due to the fact that when both limbs are equally tended the passive tension on the retractor muscles of both limbs reaches the itical value sooner than when one limb is retracted and the other protracted. The nsion on the muscles is partly due to the movement of the plantar supports away om the centre of gravity of the body; as soon as one limb is protracted most of the eight falls on this foot and the tension of the muscles in the other limb is reduced, d may not reach its critical value until the limb is fully retracted.

Although both hindlimbs of a spinal toad never step simultaneously, they never- eless appear to preserve a definite degree of independence; this has been observed nen differential rates of passive retraction are applied to the two limbs by placing e on a faster moving platform than the other. Under such conditions the frequency stepping on each limb was closely proportional to the rate of passive retraction plied (Table I). In one preparation, the left limb performed ten steps in 4 min. sec. when the drum surface was moving at 14·4 cm. per minute, whereas the ght limb performed twenty-nine steps in the same period of time on a drum rface moving at 43 cm. per minute; the right limb remained stationary when its um was stopped, and during this time the left limb maintained its customary equency on the slower moving drum.

Table I

Period of observation	Number of steps		
	Left hindlimb on drum moving at 14·4 cm./min.	Right hindlimb	
		On drum moving at 43 cm./min.	On stationary drum
min. sec.			
4 10	10	26	—
4 45	10	—	0
4 35	10	29	—
4 37	10	—	0

In these cases the frequency of stepping in one hindlimb clearly did not sub- ntially affect the frequency of stepping in the contralateral limb.

In relatively fatigued preparations a considerable latent period may elapse tween the moment of complete passive retraction of a limb and the onset of otraction and both limbs may be fully retracted simultaneously. In such case, protraction in one limb was rapidly followed by protraction in the other, suggestin that, after a period of reflex inhibition, the protractor centres were excited b proprioceptor stimulation which otherwise was inadequate to discharge the pro tractor motor centres (Fig. 3).

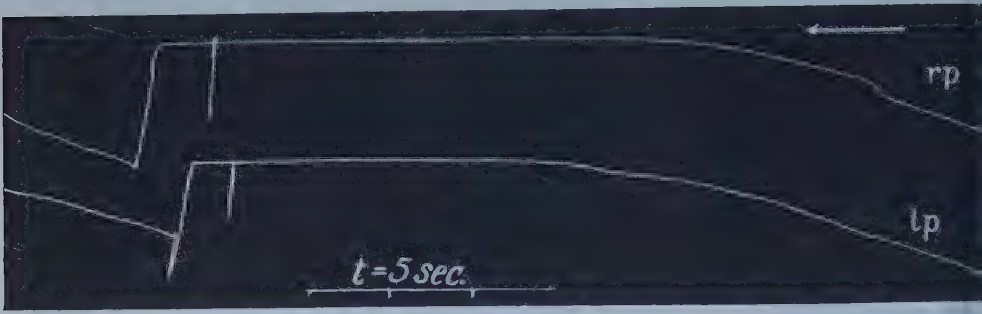

Fig. 3. Response of hindlimbs of a fatigued preparation of a spinal toad to simultaneous passi retraction. Note that the left hindlimb (*lp*) responded after a latent period of nearly 20 sec., an was quickly followed by the right hindlimb (*rp*).

The above facts seem to justify two conclusions: (*a*) protractor activity in on hindlimb inhibits protractor activity in the contralateral limb; (*b*) after a period o reflex inhibition the sensitivity of the protractor centres to proprioceptor stimulatio is increased. Subject to these limitations, each hindlimb appears to respon independently to passive retraction.

RESPONSE TO SIMULTANEOUS PASSIVE RETRACTION OF ALL FOUR LIMBS

When all four limbs of a suitable[1] preparation are in contact with the surface o a moving drum, they show, extremely clearly, the same type of diagonal co ordination which characterizes ambulation in the normal intact animal; spina preparations of this type "step" persistently for long periods of time if the speed o the drum is suitably adjusted. As soon as the drum stops, the limbs cease to move when the drum is restarted the limbs again begin to step. Equally clear and co ordinated quadripedal stepping can be elicited by towing the preparations over suitable surface. Some preparations, e.g. that shown in Fig. 4, show the diagon pattern of limb movement with little or no modifications: in the case of *Trito* however, minor modifications have, not infrequently, been observed: (*a*) one lim may miss a step from time to time; (*b*) the hind member of a diagonal pair ma be protracted before its anterior partner. In no case, however, is the basic diagon co-ordination seriously obscured.

The co-ordinated response of a diagonal limb to stimulation applied to the othe nember of the pair was recorded by Luchsinger (1880) in urodeles and other ertebrates (Guillebeau & Luchsinger, 1882); in a postural form it is well known in nammals (see Fulton, 1938). It is important to note, however, that, in slowly tepping spinal preparations of amphibia the two members of each diagonal pair lo not step simultaneously, there is a definite, although short, latent period between he protraction of the two limbs. In nearly every case the forelimb moves before he hindlimb as in normal ambulation by the intact animal, but in spinal preparaions of *Triton* the order is occasionally reversed. This phenomenon has not been

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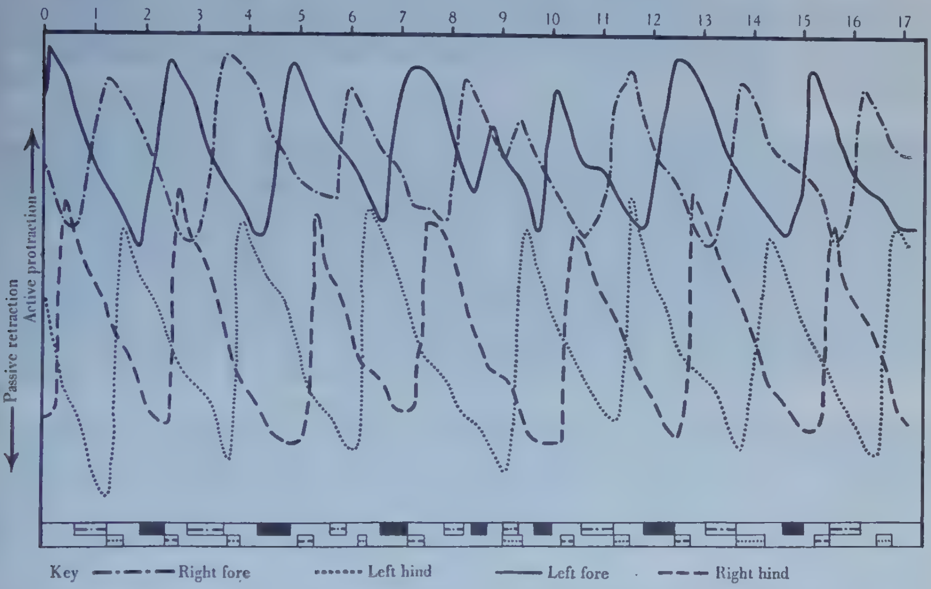
[1] After spinal transection, at the level of the first vertebra, the behaviour of the forelimbs amphibia is somewhat variable. In some cases no reflex activity has been observed; whilst in *Ra* the forelimbs not infrequently show, on mechanical stimulation, a persistent clasping reflex. I other instances, however, the forelimbs of the newt, frog and toad showed clearly defined protract responses to passive retraction. Only the latter type of preparation is suitable for the present stud



ig. 4. Graph, derived from a cinematograph record, of the diagonal stepping of a spinal toad when ll four limbs are in contact with the surface of a moving drum. Note that the diagonal pattern is reserved except at 9 sec. when both hindlimbs missed a step. The co-ordination between the nembers of the two diagonals can be followed from the record of the protractor phases shown at ne bottom of the graph.

bserved in the toad or frog, and in these forms there is definite evidence for the iew that the forelimbs, normally, act as time signals for the diagonal hindlimbs.

The diagonal pattern of limb protraction seen in spinal preparations only occurs vhen all four limbs are in contact with platforms moving at the same rate or when ne preparation is passively towed along a straight line. If a spinal newt be towed long the circumference of a circle, each of the offside limbs steps more frequently nan its near-side fellow. Each limb is protracted whenever it is fully retracted, and nus the frequency of stepping in each limb is different and depends on the time equired for passive retraction, and by varying the direction along which the animal towed, the frequency of stepping in each limb can be controlled: in all cases, owever, protraction of one limb occurs when its fellow limb at the same level of ne body is being passively retracted or is at rest.

These facts indicate fairly clearly that the diagonal pattern of stepping is the expression of four protractor reflexes, one appertaining to each limb, which are co-ordinated to form a specific diagonal pattern. It is important to note, however, that the type of co-ordination is flexible in the sense that, under appropriate conditions, each limb can be protracted without inducing a corresponding movement in the diagonal limb.

INFLUENCE OF THE FORELIMBS ON THE STEPPING OF THE HINDLIMBS OF ANURANS

When all four limbs of a spinal toad or frog are stepping on a moving drum, the frequency of step in each limb is typically the same although the length of the fully retracted hindlimbs is considerably greater than that of the forelimbs. Since the

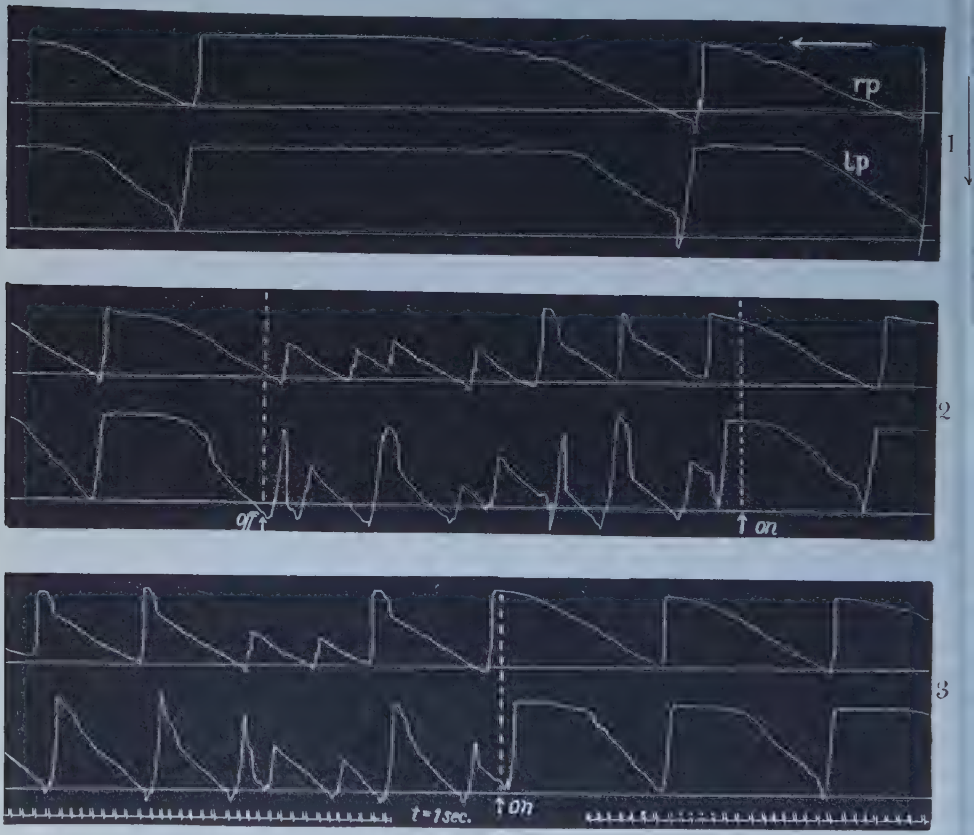
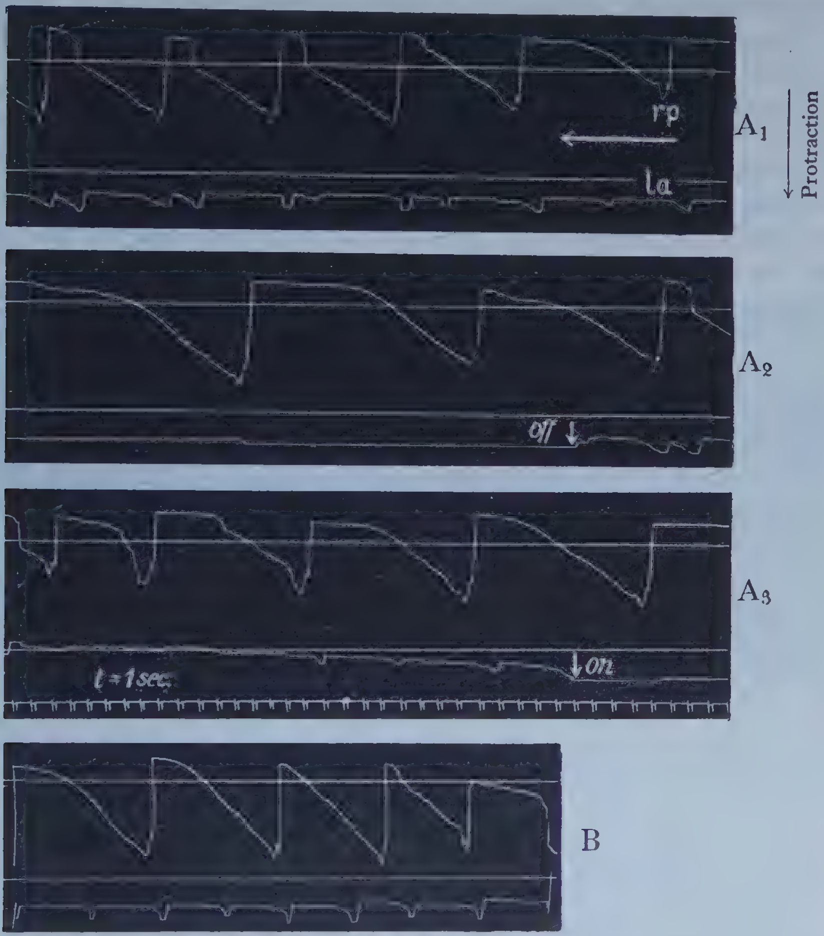


Fig. 5. Record showing the effect of reflex protraction of the forelimbs on the frequency of stepping of the hindlimbs of a spinal frog. In the top section of the tracing the forelimbs were not in contact with the drum—note the low frequency of stepping of hindlimbs: between the arrows in section 2 of the record, the forelimbs were in contact with the drum and exhibited stepping movements—note increase in frequency of stepping of hindlimbs. Subsequent removal of forelimbs from the drum induced marked reduction in frequency of stepping of hindlimbs; the frequency again rose when the forelimbs were replaced on the drum. The three sections together form a continuous record. *rp*, tracing of right posterior limb; *lp*, tracing of left posterior limb.

equency of stepping is the same for both pairs it follows that the hindlimbs must e protracted before their retraction is complete unless the forelimbs drag, prior to otraction, for a finite period at the position of full retraction. In active prepara- ns no such drag occurs and the length of step of the hindlimbs is substantially creased (from 7·5 cm. to 11 cm. in the cases examined) by placing the forelimbs a stationary support.



g. 6. A. 1–3. Three sections of a continuous record showing the effect of protraction of the left elimb (*la*) of a frog on the activity of the right hindlimb (*rp*). In section 1 all four limbs were in ntact with the surface of a moving drum; note that protraction of the forelimb usually causes re- ction of the hindlimb if the former occurs before passive retraction of the hindlimb is complete, t causes protraction of the hindlimb if the passive retraction of the hindlimb is nearly complete. section 2 of the record the forelimbs were removed from the drum surface; note slowing of the thm of the hindlimb. In section 3 the forelimbs were replaced on the drum. Tracing B shows response of a relatively fatigued preparation; note that the hindlimb responds by protraction every alternate protraction of the diagonal forelimb.

Besides altering the degree of retraction in the hindlimbs requisite for the protractor response, the activity of the forelimbs has a definite effect on the frequenc of stepping of the hindlimbs. In all cases the movement of the forelimbs accelerat the frequency of the hindlimbs; and in at least two cases, in which passive retractio of the hindlimbs alone produced no response, well-defined stepping set in as soo as the forelimbs were removed from their stationary support and placed on th moving drum. In such cases, it is usual to find that protraction of a diagona hindlimb occurs immediately after the protraction of the contralateral forelim (see Fig. 6). To this rule, however, there is one exception. If the contralateral forelimb is protracted considerably before the hindlimb is fully extended, the latter lim tends to show a marked slip over the surface of the drum—as though the retracto muscles were activated or the protractor tone reduced (see Fig. 6 A_1); in som preparations, however, this response was absent and the hindlimb was protracte only when near its own position of complete extension, i.e. at every alternat protraction of the contralateral forelimb (see Fig. 6 B).

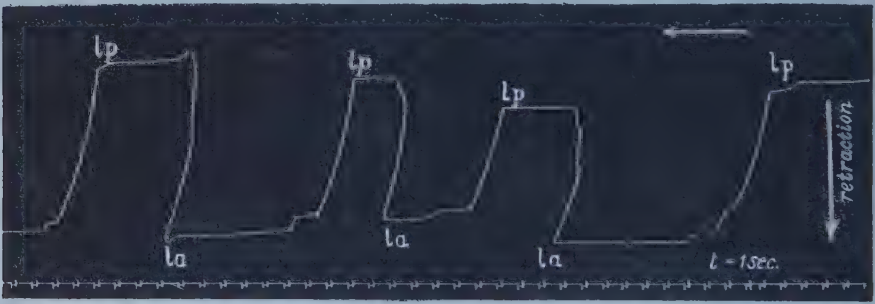


Fig. 7. Response of deafferentated right posterior limb of *Bufo* to tactile stimulation applied t deefferentated left posterior (*lp*) or deefferentated left anterior limb (*la*). On stimulating left posterio limb the right posterior limb is retracted; on stimulating left anterior limb the right posterior lim is protracted.

The excitatory effect of protraction in a forelimb on the activity of a contralatera hindlimb is well seen when the two left limbs of a preparation are stepping on drum which is moving considerably faster than that supporting the two right limbs Under such conditions the frequency of stepping of the right hindlimb is the sam as that of the left hindlimb, but the hindlimb is protracted long before the positio of full passive retraction, i.e. it "steps short".

The obvious interpretation of these observations is provided by the assumptio that a stimulus arising in the proprioceptor end organs of a forelimb spreads to the motor centres of the protractor muscles of the contralateral hindlimb; if these centres are also being exposed to subliminal excitation from its own proprioceptor organs—protraction results. If it falls on the hindlimb centres when this limb is protracted—it is either inoperative or tends to inhibit protractor tone.

The response of a limb to a protractor stimulus applied to the other member o its diagonal does not depend upon the integrity of the sensory nerve supply from the former limb. This fact is illustrated by Fig. 7 in which a protractor stimulus applied to the left forelimb of an intact toad induced protraction of the deafferentated right posterior limb.

Omitting the effect of protraction in a forelimb on a diagonal posterior limb already in the position of partial or complete protraction, the pattern of protractor reflexes seen in spinal amphibia has been incorporated into Fig. 10.

RETRACTOR REFLEXES IN SPINAL AND IN INTACT AMPHIBIA

Whereas a spinal newt, when towed over the ground (or with all four limbs in contact with a moving drum) gives a complete and persistent picture of the protractor activities seen during the ambulation of an intact animal, no trace of activity is usually seen in the retractor muscles of the limbs and extraneous stimuli applied to one limb have, so far, failed to elicit with any regularity retractor activity in that or other limbs. Regular activity in the retractor muscles of the spinal newt have only been elicited by extraneous stimuli applied to the tail (see p. 240).

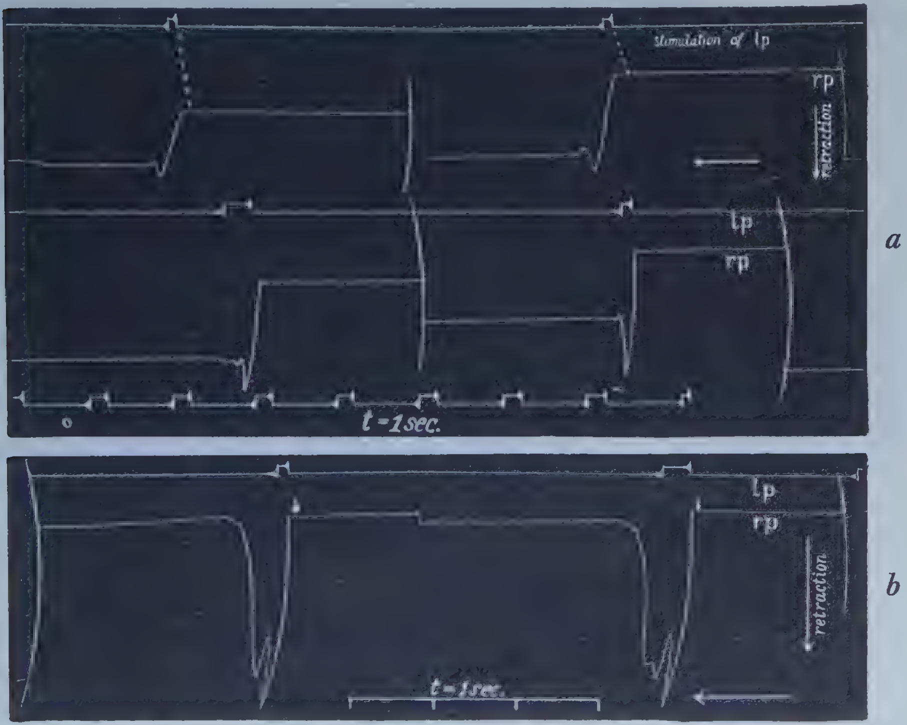


Fig. 8*a*. Crossed retractor reflex in right posterior deafferentated limb (*rp*) of *Bufo* in response to tactile stimulation of deefferentated left posterior limb (*lp*): both forelimbs were deafferentated. Note that retraction of the right posterior limb is not followed by protraction.

Fig. 8*b*. Crossed retractor reflex in right posterior intact limb (*rp*) of *Bufo* in response to tactile stimulation of deefferentated left posterior limb (*lp*). Both forelimbs were deafferentated. Note that initial retraction of right posterior limb is followed by protraction.

A spinal frog or toad differs from the spinal newt by the display of a definite degree of co-ordinated retractor activity although as a rule it is not sufficiently powerful to display itself, without special precautions, when the limbs are stepping on a moving drum.

If one limb of a spinal frog or toad be sharply seized by a pair of forceps, that limb is quickly protracted, and at the same time the contralateral limb is quickly extended (see Biedermann, 1900). If the stimulus be transitory, the contralateral limb quickly regains the protracted condition. The response of the contralateral limb is best studied in spinal preparations in which the motor roots of one hindlimb (the left), and the dorsal roots of the other limb (the right) have been cut. At the beginning of the experiment the left hindlimb is placed in the fully or partially retracted position, and the right limb is placed in a fully protracted posture: if the left limb is now tapped with a seeker, the right limb is immediately retracted and remains retracted (Fig. 8*a*). If this experiment be repeated with a preparation in which the dorsal roots of the right limb have not been cut, this limb after its active retraction returns to its normal protracted posture (Fig. 8*b*). These observations establish two facts: (*a*) that a well-defined crossed retractor reflex can be displayed by the hindlimbs of a toad or frog; (*b*) when a hindlimb is reflexly retracted it returns, as soon as the initial reflex stimulus has ceased, to the protracted position owing to the activity of its own proprioceptor mechanism. It must be remembered, however, that the crossed retractor reflex does not, in our experience, readily display itself in response to such stimuli as are applied to the limb by a moving drum whose surface is sufficiently rough to effect passive retraction of the limb. We have, how-

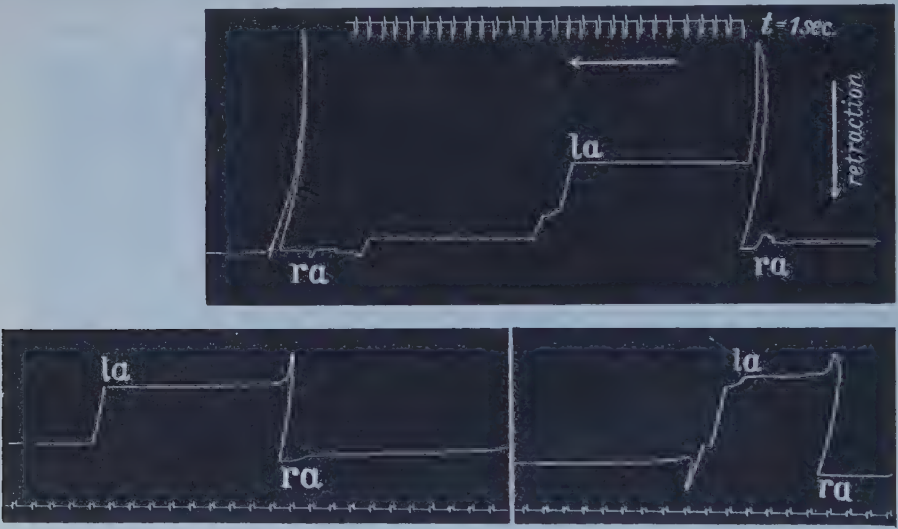


Fig. 9. Response of deafferentated left posterior limb of *Bufo* to tactile stimulation applied to deefferentated right anterior limb (*ra*) or left anterior limb (*la*). On stimulating left anterior limb the left posterior limb retracts; on stimulating the right anterior limb the left posterior limb is protracted, but this is sometimes followed by retraction.

ever, observed that reflex retraction of a hindlimb accompanies reflex protraction of an ipsilateral forelimb when the forelimbs are in contact with a moving drum and the hindlimbs lying on a stationary smooth glass plate. Similarly normal toads, both of whose hindlimbs have been deafferentated, show clearly defined retraction of the psilateral hindlimb when the forelimb responds, by protraction, to passive retraction: simultaneously the contralateral hindlimb responds by protraction (see Fig. 9).

DISCUSSION

The picture presented by the protractor muscles, when all four limbs of a spinal amphibian are in contact with the surface of a moving drum, resembles sufficiently closely that of the same muscles during normal ambulation in the intact animal to ustify the view that ambulatory co-ordination is to be regarded as an integration of a series of reflexes, some of which arise in the proprioceptor sense organs of the imbs and adjacent structures. This conclusion is of the same essential nature as that put forward by Philippson (1905) and later by Sherrington (1910) for the dog although the nature of the fundamental reflexes is different to those described by Philippson. Philippson attributed the onset of protraction to the strong pressure which a limb exerts against the ground during the height of its retraction—the protractor phase being brought to an end by stimuli arising in the stretched skin in the inguinal region of the contralateral limb. Philippson also concluded that contact of the foot with the ground excited retractor activity as also did flexion of he knee joint of the contralateral limb.

Both Sherrington (1913) and Graham Brown (1912 *a*, *b*, 1913) marshalled trong evidence against the conclusions reached by Philippson. Sherrington showed hat desensitization of all four feet and narcotization of the inguinal skin did not nterfere with the precision of movement of a cat, and, as is well known, the hindlimbs of a spinal dog step persistently when all four limbs are suspended in air. Further, one limb may step whilst its fellow is at rest. Both Sherrington and Graham Brown showed that rhythmical movements can be induced in a pair of totally deafferentated antagonistic muscles if their motor centres are exposed simultaneously o two well-balanced streams of excitatory and inhibitory stimuli, and they therefore oncluded that the fundamental rhythm of normal ambulation was of central and not of peripheral origin.

The facts described in this paper show that a fully co-ordinated rhythm can be ustained in the protractor muscles of the limbs of spinal amphibia and that this hythm is essentially due to the interdependence of well-defined peripheral reflexes. No such rhythm has been observed in amphibia whose central nervous system has een isolated from the periphery (Gray & Lissmann, 1940). If a central rhythm, of he type envisaged by Sherrington and Graham Brown, exists in the amphibia it is ecessary to assume that the two streams of opposing stimuli necessary for its maintenance are derived from the rhythmical activity of peripheral sense organs. Under such conditions the difference between the central and peripheral concepts argely disappears. We may envisage a mechanism which when carefully balanced on the two sides yields a rhythm much as is the case in the arm of a balance with equal weights in the two pans; we can, however, oscillate the balance arms by adding or removing weights alternatively to the two sides. The latter type of control seems much more effective as a method of enabling the mechanism to respond with certainty to a variety of external forces which may be applied to one side or the other.

If we accept the view that the normal ambulatory rhythm is essentially a chain of reflexes it is easy to see how the limbs can respond in a co-ordinated manner to events taking place in sense organs other than those located in the muscles themselves; under natural conditions the movements of the limbs are influenced by visual and labyrinthine stimulation. These sense organs usually exert their effect on the limbs by inducing movements of the head relative to the shoulder, thus stretching some of the muscles of the forelimbs; this, in turn, induces movements in the limbs. To a very large extent the control of limb movements in the amphibia is exercised by proprioceptor endings in the muscles and associated structures, although, as we have shown elsewhere (Gray & Lissmann, 1940), the sensory nerve supply of only a small fraction of the musculature involved in any particular movement need be intact.

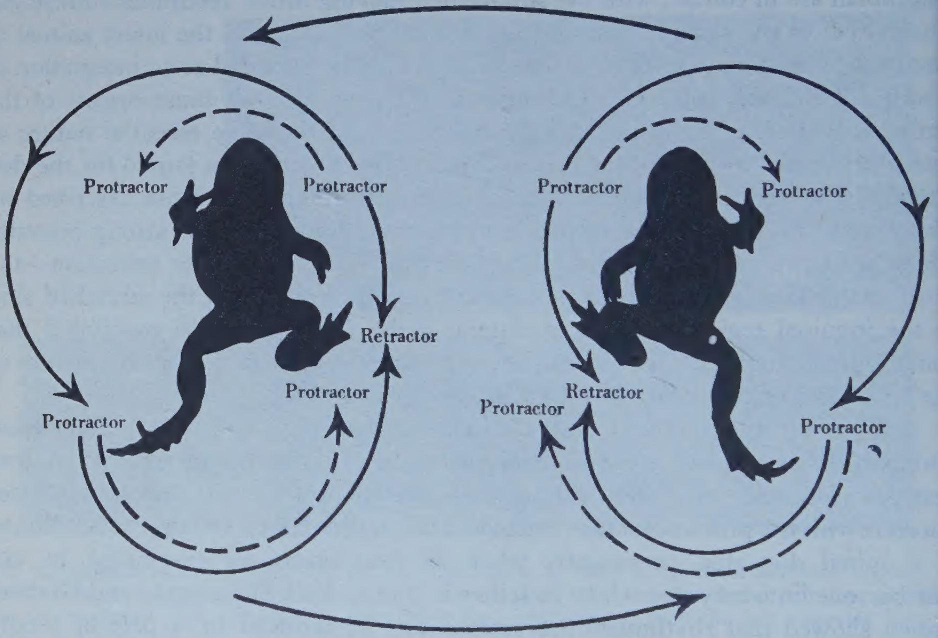


Fig. 10. Diagram illustrating the reflex pattern of stepping in an amphibian. The full lines represent excitatory effects, the dotted lines represent inhibitory effects.

The pattern of reflexes described in this paper are indicated in Fig. 10 and lead to a conception of normal ambulation which is of the same general nature as that reached by Sherrington (1910) prior to his observation of stepping in deafferentated

ammalian muscles. In the spinal amphibia, however, the response of the retrac-
on centres to reflex protraction of other limbs is not sufficient to cause active
traction of the limb against any significant amount of external resistance. In
ig. 10 the reflex pattern is shown as a closed chain capable of sustaining rhythmical
nbulation, but it remains to be seen how far the retractor centres of an intact
nimal depend on sources of excitation not indicated in the diagram or how far the
ability of the spinal preparation to walk, without continuous extraneous stimula-
on, is due to the depressant effects of spinal transection on the retractor centres.

SUMMARY

1. Individual limbs of spinal amphibians when passively retracted respond by
tive protraction.

2. When both forelimbs or both hindlimbs are simultaneously subjected to
assive retraction they respond alternately and not simultaneously.

3. When all four limbs are simultaneously subjected to passive retraction their
der of response follows the diagonal pattern typical of normal ambulation. When
l four limbs are in contact with a moving platform, the diagonal rhythm of limb
otraction is sustained for long periods.

4. In the intact toad or frog, a protractor reflex in one limb is accompanied by
traction in the ipsilateral fellow limb and by protraction of the diagonal limb.

5. The normal ambulatory cycle in amphibia is to be regarded as a series of
-ordinated reflexes, largely dependent upon proprioceptor sense organs, and not
the expression of a centrally determined rhythm.

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